DESCRIPTION

HAIRPIN HYBRIDIZER MOLECULES FOR MODULATION OF GENE EXPRESSION

Background Of The Invention

This invention relates to nucleic acid molecules, which the Applicant terms "hairpin hybridizer" (HPH) molecules that are capable of modulating gene expression by hybridizing to target RNA with improved specificity to thereby block translation of such target RNA.

The following is a discussion of relevant art, none of which is admitted to be prior art to the present invention.

Since the discovery of the mechanisms underlying gene expression, specifically nucleic acid based transcription and translation, a great deal of effort has been placed on blocking or altering these processes for a variety of purposes, such as understanding biology, gene function, disease processes, and identifying novel therapeutic targets. Approaches involving the use of nucleic acid molecules for modulating gene expression have gained popularity in recent years. For example, nucleic acid molecules have been designed which are capable of binding to specific mRNA sequences by Watson-Crick base pairing interaction and blocking translation (Crooke, 1996, *Medicinal Res. Rev.* 16, 319-344). Another approach involves complexation of DNA with triplex forming oligonucleotides to prevent transcription of bound DNA sequences thereby inhibiting gene expression (Kim *et al.*, 1998, *Biochemistry.* 37, 2299-2304). The interaction of antisense oligonucleotides, 2-5A antisense chimera, or ribozymes with target RNAs have been used to modulate gene expression. All of these nucleic acid molecules are highly specific to their matching target sequences and therefore may offer lower toxicity compared to traditional approaches such as chemotherapy.

The concept of gene expression inhibition through an antisense mechanism is derived in part from mechanisms found in nature. It was found that prokaryotic systems utilized complementary RNA molecules to inhibit translation (Lacetena *et al.*, 1983, *Blood* 170, 635-650). Simons *et al.*, 1983, *Cell* 34, 673-682; Mizuno *et al.*, 1984, *Proc. Natl. Acad Sci. (USA)* 81, 1966-1970). For example, expression of *E. coli* OmpF and OmpC genes (outer membrane proteins) is regulated by an antisense RNA mechanism (Mukopadhyay & Roth, 1996, *Critical Rev. In Oncogenesis* 7, 151-190).

Antisense oligonucleotides can be used to down-regulate target mRNA by a number of different mechanisms. The specificity of these reagents is determined by the primary sequence (GC content, sequence length), chemistry (ribonucleotides, deoxy-ribonucleotides, chemically modified nucleotides) of the antisense molecule and the presence of pseudo-target sequences. Pseudo-targets are nucleic acid sequences, which may have sequence identity or homology to a target sequence. The number of pseudo-targets for a given sequence, especially human genes, is largely unknown at this point, since only a minor fraction of the human genome is currently sequenced.

Lizardi *et al.*, US Patent No. 5,312,728, describe a self hybridizing nucleic acid molecule, referred to as a molecular switch used, for the detection of target nucleic acid molecules. The molecular switch consists of a probe sequence of 20 to 60 nucleotides capable of hybridizing to a target sequence and 5' and 3' sequences of at least 10 nucleotides in length which are capable of hybridizing to each other intramolecularly.

Engdahl *et al.*, 1997, *Nucleic Acids Research* 25, 3218-3227, describe the use of an RNA cassette system for silencing the *lacI* gene. The molecules used consisted of a hairpin structure, which was used for target sequence recognition and an inhibitor region which was either an antisense or ribozyme sequence.

Delihas *et al.*, 1997, *Nature Biotech* 15, 751-753, describe the formation of non-canonical base-pairs using natural antisense RNA and target RNA.

Stinchcomb *et al.*, International PCT Publication NO. *WO 95/23225*, describe an RNA molecule with an intramolecular stem-loop structure of greater than or equal to eight base-pairs.

Summary Of The Invention

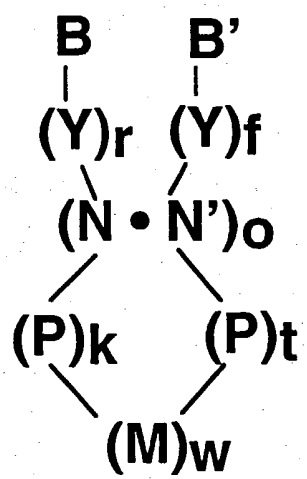
This invention relates to nucleic acid molecules capable of binding and blocking the function of target nucleic acid molecules, thereby modulating cellular or viral mechanisms including splicing, editing, replication or gene expression, and translation. Specifically, the invention concerns novel nucleic acid molecules with a hairpin secondary structure capable of down regulating protein expression by binding (steric blocker) and optionally facilitating the cleavage of target RNA through an RNase H or other mechanism. For simplicity and ease of understanding the instant invention, the nucleic acid molecules of the instant invention shall be referred to as hairpin hybridizer (HPH) molecules. In particular, applicant describes the use of these HPH molecules to down-regulate gene expression in bacterial, microbial, fungal, eukaryotic systems including plant, or mammalian cells. Down-regulation of specific target sequences may either have a therapeutic effect in many diseases or disease states or aid in the identification of gene function and/or new therapeutic gene targets. The HPH molecules of the present invention can be used for *in vitro* or *in vivo* applications well known in the art.

The present invention features a method of modulating the function of a target sequence in a cell using HPH molecules. HPH molecules include target binding region and a hairpin region, where the target binding region is capable of binding to the target sequence in a sequence specific manner *in vitro* or *in vivo* to modulate the function of the target sequence. The hairpin region of the HPH molecule provides an improved specificity characteristic to the HPH molecule. The hairpin region may also provide improved resistance to nuclease degradation, may help the HPH molecule with localization inside a cell, and may also help in improved uptake of the HPH molecule by

the cells compared to a molecule lacking such a hairpin structure. The target binding region of the HPH molecule may also include an RNase H activating region where such a region includes a greater than or equal to 4 deoxyribonucleotide nucleotide sequence with phosphorothioate, phosphodiester, phosphorodithioate, and/or 5'thiophosphate internucleotide linkages. The RNase H activating region interacts with the target RNA to form a DNA:RNA complex which is recognized by the cellular RNase H enzyme, which binds the DNA:RNA complex and cleaves the RNA portion of the DNA:RNA complex. Such cleavage of the target RNA by RNase H causes the target RNA to lose its normal function by causing inhibition of its translation into proteins, its replication, its packaging into viral particles, or other functions.

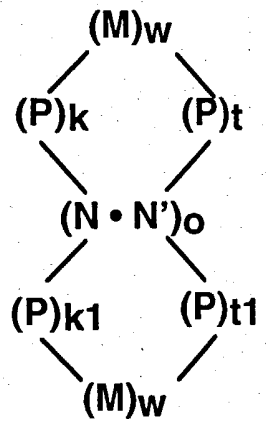
Thus in a first aspect, a method of modulating the function, such as expression, of a target sequence, preferably in a cell, comprising the step of contacting said target sequence with a hairpin hybridizer nucleic acid molecule under conditions suitable for the modulation of said target sequence expression, wherein said hairpin hybridizer nucleic acid molecule consists of the following formula:

Formula I:



where, each P, Y, N and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 8 or 9; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4 and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; $(P)_t$ and $(P)_k$ are independently oligonucleotides, preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); $(P)_t$ and $(P)_k$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate methylphosphonate linkers and the like or a combination thereof; k and t may be the same length (k=t) or different lengths ($k \neq t$); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers include phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length ($k = t = w$) or different length ($k \neq t \neq w$) or ($k = t \neq w$) or ($k \neq t = w$) or ($k = w \neq t$); at least one or more of each said (P)t, (P)k, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); r and f are independently an integer greater than or equal to zero, specifically 1, 2, 3, 4, 5, 10, or 15; each B and B' independently represents a cap structure which may independently be present or absent; when r= 0, and f=0, B and/or B', when present, is attached to $(N•N')_o$; and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate or others known in the art).

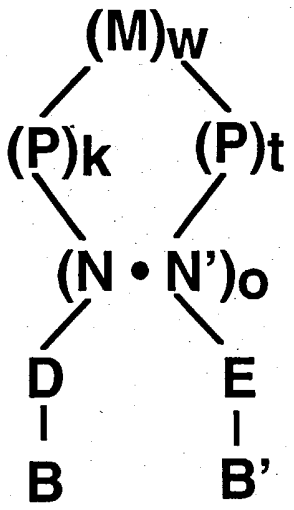
Formula II:



where, each P, N, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 8 or 9; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; k1 is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t1 is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4 and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; $(P)_t$ and $(P)_k$, $(P)_{t1}$, and $(P)_{k1}$ are independently oligonucleotides, preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); $(P)_t$ and $(P)_k$, $(P)_{t1}$, $(P)_{k1}$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate linkers and the like or a combination thereof; k and t may be the same length (k=t) or different lengths ($k \neq t$); k1 and t1 may be the same length (k1=t1) or different lengths ($k \neq t$); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers include phosphodiester,

phosphorothioate, 5'thiophosphate, methylphosphonate, or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length (k = t = w) or different length (k ≠ t ≠ w) or (k = t ≠ w) or (k ≠ t = w) or (k = w ≠ t); t1, k1, and w may be of the same length (k1 = t1= w) or different length (k1 ≠ t1 ≠ w) or (k1 = t1 ≠ w) or (k1 ≠ t1 = w) or (k1 = w ≠ t1); at least one or more of each said $(P)_t$, $(P)_k$, $(P)_{t1}$, $(P)_{k1}$ and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate, or others known in the art).

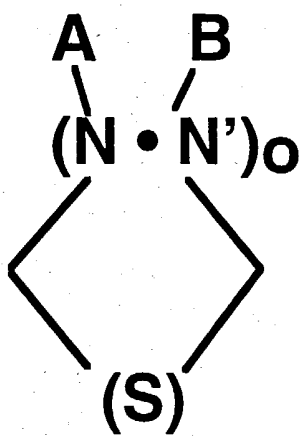
Formula III:



where, each P, N, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 8 or 9; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4

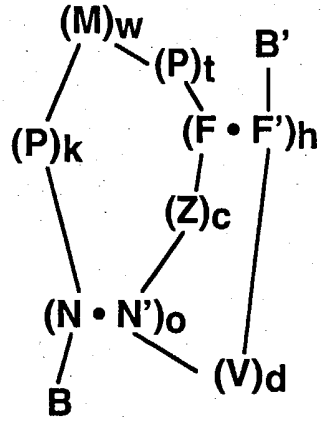
and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; $(P)_t$ and $(P)_k$ are oligonucleotides preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); each $(P)_t$ and $(P)_k$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate linkers and the like or a combination thereof; k and t may be the same length (k=t) or different lengths ($k \neq t$); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers may include phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate, or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length ($k = t = w$) or different length ($k \neq t \neq w$) or ($k = t \neq w$) or ($k \neq t = w$) or ($k = w \neq t$); at least one or more of each said (P)t, (P)k, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); D and E are oligonucleotides which are greater than or equal to 4 and preferably less than 100 nucleotides in length, more specifically 6, 7, 8, 9, 10, 11, 12, 15, 20, or 30 and are of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); The D and E oligonucleotides may be symmetric ($D = E$ in length) or asymmetric ($D \neq E$ in length); each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate or others known in the art).

Formula IV:



where, N represents a ribonucleotide which may be the same or different; N' is a nucleotide complementary to N; • indicates hydrogen bond formation between two adjacent ribonucleotides; o is an integer greater than or equal to 3 and less than or equal to 9, more specifically 4, 5, 6, 7, 8 or 9; S, A, and B are oligoribonucleotides which are independently equal to 5 and preferably less than 100 nucleotides in length, more specifically 6, 7, 8, 9, 10, 11, 12, 15, 20, or 30; S is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); and _____ represents a phosphodiester linkage.

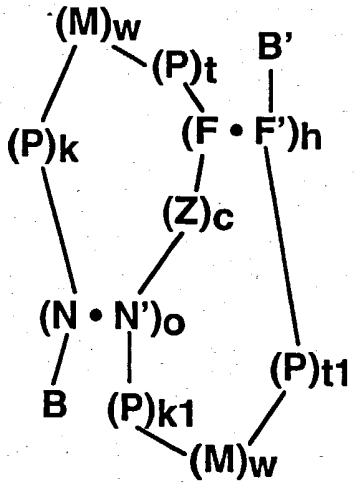
Formula V:



where, each P, N, F, V, Z, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide complementary to F; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, or 15; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4 and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; d is an integer greater than or equal to 3 and preferably less than about 20, more specifically 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, or 18; h is an integer greater than or equal to 2 and preferably less than about 10, more specifically 2, 3, 4, 5, 6, 7, 8, or 9; c is an integer greater than or equal to 0 and preferably less than about 20, more specifically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, or 18; $(P)_t$, $(P)_k$, $(P)_{tl}$, $(P)_{kl}$, $(V)_d$ and $(Z)_C$ are oligonucleotides preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); each $(P)_t$, $(P)_k$, $(P)_{tl}$, $(P)_{kl}$, $(V)_d$ and $(Z)_C$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate linkers and the like or a combination thereof; k and

t may be the same length (k=t) or different lengths (k ≠ t); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers may include phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate, or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length (k = t = w) or different length (k ≠ t ≠ w) or (k = t ≠ w) or (k ≠ t = w) or (k = w ≠ t); at least one or more of each said (P)t, (P)k, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate or others known in the art). In a preferred embodiment N and/or N' in $(N•N')_o$, F and/or F' in $(F•F')_h$ and/or $(Z)_c$, may optionally be able to independently interact with a target sequence.

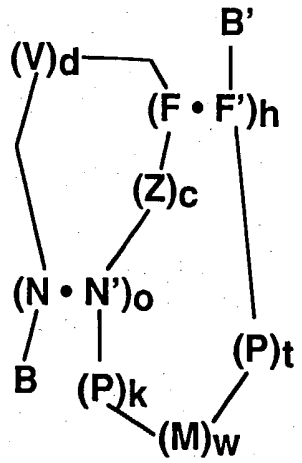
Formula VI:



where, each P, N, F, Z, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide complementary to F; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 9, 10, 11, 12, or 15; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more

specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; k1 is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t1 is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4 and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; h is an integer greater than or equal to 2 and preferably less than about 10, more specifically 2, 3, 4, 5, 6, 7, 8, or 9; c is an integer greater than or equal to 0 and preferably less than about 20, more specifically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, or 18; $(P)_t$,$(P)_k$, and $(Z)_C$ is an oligonucleotide preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); each $(P)_t$,$(P)_k$, and $(Z)_C$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate linkers and the like or a combination thereof; k and t may be the same length (k=t) or different lengths ($k \neq t$); k1 and t1 may be the same length (k1=t1) or different lengths ($k1 \neq t1$); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers may include phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length ($k = t = w$) or different length ($k \neq t \neq w$) or ($k = t \neq w$) or ($k \neq t = w$) or ($k = w \neq t$); t1, k1, and w may be of the same length ($k1 = t1 = w$) or different length ($k1 \neq t1 \neq w$) or ($k1 = t1 \neq w$) or ($k1 \neq t1 = w$) or ($k1 = w \neq t1$); at least one or more of each said $(P)_t$, $(P)_k$, $(P)_{t1}$, $(P)_{k1}$,and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate or others known in the art). In a preferred embodiment N and/or N' in $(N•N')_o$, F and/or F' in $(F•F')_h$ and/or $(Z)_c$, may optionally be able to independently interact with a target sequence.

Formula VII:



where, each P, N, F, V, Z, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide complementary to F; o is an integer greater than or equal to 3, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, or 15; k is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; t is zero or an integer greater than or equal to 3 and preferably less than about 100, more specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, or 20; w is an integer greater than or equal to 4 and preferably less than 100, more specifically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20; d is an integer greater than or equal to 3 and preferably less than about 20, more specifically 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, or 18; h is an integer greater than or equal to 2 and preferably less than about 10, more specifically 2, 3, 4, 5, 6, 7, 8, or 9; c is an integer greater than or equal to 0 and preferably less than about 20, more specifically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, or 18; $(P)_t$,$(P)_k$, $(V)_d$ and $(Z)_C$ are oligonucleotides preferably including at least one position that is not deoxynucleotide (e.g. 2'-H containing nucleotide); each $(P)_t$,$(P)_k$, $(V)_d$ and $(Z)_C$ may include phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate linkers and the like or a combination thereof; k and t may be the same

length (k=t) or different lengths (k ≠ t); $(M)_w$ is an oligonucleotide sequence whose inter-nucleotide linkers may include phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate, or phosphorodithioate linkers or a combination thereof; t, k, and w may be of the same length (k = t = w) or different length (k ≠ t ≠ w) or (k = t ≠ w) or (k ≠ t = w) or (k = w ≠ t); at least one or more of each said (P)t, (P)k, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence (the target can be an RNA, DNA or RNA/DNA mixed polymers); each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage (*e.g.* a phosphate ester linkage, amide linkage, phosphorothioate, phosphorodithioate, 5'-thiophosphate, methylphosphonate or others known in the art). In a preferred embodiment N and/or N' in $(N•N')_o$, F and/or F' in $(F•F')_h$ and/or $(Z)_c$, may optionally be able to independently interact with a target sequence.

In a preferred embodiment, the invention features an HPH molecule of any of formulae I-III, and V-VII, where the $(M)_w$ optionally includes an RNase H activating region. By "RNase H activating region" is meant, a region capable of binding to a target RNA to form, for example, a $(M)_w$• target RNA complex that is recognized by cellular RNase H, where the RNase H enzyme will then bind to the $(M)_w$• target RNA complex and cleave the target sequence.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187;

Uhlman & Peyman, *supra*) all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

By "ribonucleotide" is meant a nucleotide with one of the bases adenine, cytosine, guanine, or uracil joined to the 1' carbon of β-D-ribo-furanose.

By "unmodified nucleotide" is meant a nucleotide with one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleotide" is meant a nucleotide that contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

By "abasic" is meant nucleic acid sugar moieties lacking a base or having other chemical groups in place of base at the 1' position.

By "sufficient length" is generally meant an oligonucleotide of greater than or equal to 4 nucleotides, or an equivalent chemical moiety able to bind and interact with a target nucleic acid molecule in solution and/or in a cell under physiological conditions.

By "complementary" or "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with another RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

By "stably interact" is meant an interaction of the oligonucleotides with target nucleic acid (*e.g.*, by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions). The term shall also mean the interaction of HPH molecules with the target molecule for a duration, under physiological conditions, in solution or in a cell, sufficient for the HPH molecule to interfere with the function of the target nucleic acid molecule.

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be composed of modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "inhibit" is meant, an impediment to normal function of a macromolecule caused by the introduction a foreign substance.

By "target sequence" or "target nucleic acid molecule" is meant, a gene or partial sequence thereof, and those elements necessary for its expression, regulation, or its transcription or replication product or intermediates or portions thereof, including DNA, RNA or protein.. Non-limiting examples of target sequence include c-raf mRNA, hepatitis C RNA, vascular endothelial growth factor receptor (*e.g.*, flt- and KDR), ras RNA, and the like.

By "antisense" is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm *et al.*, 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review see Stein and Cheng, 1993 *Science* 261, 1004).

By "cap structure" is meant chemical modifications which have been incorporated at the terminus of the oligonucleotide (*e.g.*, B and B' in formulae I-III). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell.

In another preferred embodiment $(P)_k$, $(P)_t$, $(N•N')_o$, $(F•F')_h$, $(V)_d$, $(Z)_c$ $(P)_{kl}$, $(P)_{tl}$, $(M)_w$, $(Y)_r$, $(Y)_f$, D and/or E, independently include modifications selected from a group comprising 2'-*O*-alkyl (*e.g.* 2'-*O*-allyl; Sproat *et al., supra*) sometimes referred to as RNA modifications; 2'-*O*-alkylthioalkyl (*e.g.* 2'-*O*-methylthiomethyl; Karpeisky *et al.*, 1998, *Nucleosides & Nucleotides* 16, 955-958); L-nucleotides (Tazawa *et al.*, 1970, *Biochemistry* 3499; Ashley, 1992, *J. Am. Chem. Soc.* 114, 9731; Klubmann *et al.*, 1996, Nature Biotech 14, 1112); 2'-*C*-alkyl (Beigelman *et al.*, 1995, *J. Biol. Chem.* 270, 25702); 1-5-Anhydrohexitol; 2,6-diaminopurine (Strobel *et al.*, 1994, *Biochem.* 33, 13824-13835); 2'-(*N*-alanyl) amino-2'-deoxynucleotide; 2'-(*N*-beta-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-*O*-amino (Karpeisky *et al.*, 1995, *Tetrahedron Lett.* 39, 1131); 2'-deoxy-2'-(*N*-histidyl) amino; 5-methyl (Strobel, *supra*); 2'-(*N*-b-carboxamidine-beta-alanyl)amino; 2'-deoxy-2'-(N-beta-alanyl) (Matulic-Adamic *et al.*, 1995, *Bioorg. & Med. Chem. Lett.* 5,2721-2724); xylofuranosyl (Rosemeyer *et al.*, 1991, *Helvetica Chem. Acta,* 74, 748; Seela *et al.*, 1994, *Helvetica Chem. Acta,* 77, 883; Seela *et al.*, 1996, *Helvetica Chem. Acta,* 79, 1451).

In yet another preferred embodiment B' is selected from a group comprising inverted abasic residue,. 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging

methylphosphonate moiety (for more details see Beigelman *et al.*, International PCT publication No. WO 97/26270, incorporated by reference herein).

In yet another preferred embodiment B is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moeity; 5'-5'-inverted abasic moeity; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

In a preferred embodiment, the HPH molecules including the molecules described in formulae I-VII are capable of binding to the target nucleic acid molecules in a sequence specific manner. The stable interaction between the HPH molecule and the target molecules interferes with the normal function of the target molecule. Such interaction, for example, may cause inhibition of the function of the target molecule, such as transcription, translation, and replication. The HPH molecules of the invention interact and interfere with the target molecule *in vitro* or *in vivo* in a bacterial cell, microbial system, plant system, or mammalian system to modulate the function of the target molecule in such biological systems. In a preferred embodiment, the HPH molecules of the instant invention are used to inhibit target gene expression in a biological system, more specifically in a cell, tissue, organ, and animal.

In a preferred embodiment, the HPH nucleic acid molecules including the molecules of formulae I-VII are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers.

In another aspect of the invention, the HPH nucleic acid molecules described in formulae IV are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. HPH molecule expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the HPH molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of HPH nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecules bind to target mRNA. Delivery of nucleic acid molecules expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510). In another aspect of the invention, nucleic acid molecules that bind target molecules and inhibit cell proliferation are expressed from transcription units inserted into DNA, RNA, or viral vectors. Preferably, the recombinant vectors capable of expressing the HPH molecules are locally delivered as described above, and transiently persist in smooth muscle cells. However, other mammalian cell vectors that direct the expression of RNA may be used for this purpose.

By "phenotype" is meant, the entire physical, biochemical, and physiological makeup of an organism as determined both genetically or environmentally and any one or any group of such traits.

In a preferred embodiment, the 5' and/or 3' portions of the hairpin region of the HPH molecule is independently complementary to the target sequence. Specifically, N and/or N' portion of the $(N•N')_o$ in formulae I-VII is independently complementary to the target sequence.

In a preferred embodiment, the 5' and/or 3' portions of the hairpin region of the HPH molecule is independently complementary to the target sequence. Specifically, N and/or N' portion of the $(F•F')_h$ in formulae I-VII is independently complementary to the target sequence.

In a preferred embodiment, the invention features a method of modulating the function of a target sequence including the steps of contacting the target sequence with the HPH molecules, including the molecules of formulae I-VII, under conditions suitable for the modulation of the function of the target sequence. Such modulation can take place *in vitro* or *in vivo*, in microbial, plant, or mammalian systems where the modulation of function may include inhibition of gene expression, modification of cellular function, change in the organism's phenotype, inhibition of replication of a virus and/or viral RNA, inhibition of motility, migration of a cell and others.

By "patient" is meant an organism that is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which enzymatic nucleic acid molecules can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

Description Of The Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1 is a schematic representation of the binding of the hairpin hybridizer (HPH) molecule to a target RNA. During binding, both the 5' and 3' sequences of the hairpin region may be non-complementary to the target sequence. Alternatively, either the 5' or 3' sequence may be complementary to the target RNA molecule independently.

Figure 2A displays the hairpin structure of the unbound HPH nucleic acid molecule including a 4 base pair stem and an internal 9 nucleotide DNA sequence. The figure further displays the structure of the nucleic acid molecule before and after binding to RNA. This molecule's 5' and 3' sequences form the hairpin structure but do not base pair with the target RNA molecule. Figure 2B displays the hairpin structure of the unbound nucleic acid molecule also including a 4 base pair stem and an internal 9 nucleotide DNA sequence. This molecule's 5' and 3' sequence forms the hairpin structure. In certain embodiments, the 5' and/or 3' sequence is capable of binding to the target RNA molecule independently.

Figure 3 displays non-limiting structures of the HPH molecules that are within the scope of the present invention. Figure 3A represents a circular nucleic acid molecule with an internal hairpin structure, each loop within the molecule is capable of binding to a target sequence; Figure 3B represents a molecule comprised of DNA and non-DNA nucelotides with an internal hairpin, target nucleic acid sequence binding region, and additional nucleotide sequences at the 5' and 3' ends of the hairpin structure which are of equal length that may optionally bind to target sequences; Figure 3C represents a molecule comprised of DNA and non-DNA nucleotides with an internal hairpin, target nucleic acid sequence binding region, and additional nucleotide sequences at the 5' end of the hairpin structure that may optionally bind to target sequences; Figure 3D represents a molecule comprised of DNA and non-DNA nucleotides with an internal hairpin, target nucleic acid sequence binding region, and additional nucleotide sequences at the 3' end of the hairpin structure that may optionally bind to target sequences; Figure 3E represents a molecule comprised of DNA and non-DNA nucleotides with an internal hairpin, target

nucleic acid sequence binding region, and additional nucleotide sequences at the 5' and 3' ends of the hairpin structure which are asymmetric in length and which may optionally bind to target sequences; Figure 3F represents a discontinuous circular nucleic acid molecule with an internal hairpin structure, each loop within the molecule is capable of binding to a target sequence.

Figure 4 displays a graph demonstrating the effect of a 31mer HPH nucleic acid molecule of the present invention on reducing c-raf mRNA levels in PC-3 cells compared to untreated and mismatch controls. The cells were treated with nucleic acid molecules for 1, 3, or 5 days and then harvest to quantify the c-raf RNA.

Figure 5 displays a graph demonstrating the effect of a 33mer nucleic acid molecule of the present invention on reducing c-raf mRNA levels in PC-3 cells compared to untreated and mismatch controls. The cells were treated with the HPH nucleic acid molecules for 1, 3, or 5 days and then harvested to quantify the c-raf RNA.

Figure 6 displays a graph demonstrating the effect of a 35mer HPH nucleic acid molecule of the present invention on reducing c-raf mRNA levels in PC-3 cells compared to untreated and mismatch controls. The cells were treated with nucleic acid molecules for 1, 3, or 5 days and then harvest to quantify the c-raf RNA.

Figure 7 displays a graph demonstrating the effect of a 31mer HPH nucleic acid molecule of the present invention on reducing c-raf mRNA levels in PC-3 cells compared to a mismatch control.

Figure 8 displays a graph demonstrating the effect of a 31mer HPH linear antisense molecule on reducing c-raf mRNA levels in PC-3 cells compared to a mismatch control.

Figure 9 displays the HPH nucleic acid molecule-based specific inhibition of c-raf RNA levels in PC-3 cells and the effect of 1, 2 and 4 base mismatches on this inhibition.

Figure 10 displays several non-limiting examples of psuedoknot hairpin hybridizer molecules. Figure 10A is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, and a target binding sequence located in closer proximity to the 5' end of the nucleic acid molecule compared to the 3' end. Figure 10B is a psuedoknot hairpin

hybridizer molecule comprised of 2 hairpin structures, and a target binding sequence located in closer proximity to the 3' end of the nucleic acid molecule compared to the 5' end. Figure 10C is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, and two target binding sequences. Figure 10D is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, a target binding sequence located in closer proximity to the 3' end of the nucleic acid molecule compared to the 5' end, and additional nucleotide sequences attached at the 5' and 3' ends of the hairpin hybridizer molecule. These additional sequences may be of equal or unequal length. Figure 10E is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, 2 target binding sequences, and additional nucleotide sequences attached at the 5' and 3' ends of the hairpin hybridizer molecule. These additional sequences may be of equal or unequal length. Figure 10F is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, a target binding sequence located in closer proximity to the 5' end of the nucleic acid molecule compared to the 3' end, and an additional nucleotide sequence attached at the 5' of the hairpin hybridizer molecule. Figure 10G is a psuedoknot hairpin hybridizer molecule comprised of 2 hairpin structures, a target binding sequence located in closer proximity to the 5' end of the nucleic acid molecule compared to the 3' end, and additional nucleotide sequences attached at the 5' and 3' ends of the hairpin hybridizer molecule. These additional sequences may be of equal or unequal length.

Mechanism of action of The HPH Nucleic Acid Molecules of the Invention

Antisense molecules known in the art are usually RNA or DNA oligonucleotides and primarily function by specifically binding to complementary (matching) sequences resulting in inhibition of peptide synthesis (Wu-Pong, Nov 1994, *BioPharm*, 20-33). The oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences by either steric blocking or RNase H mediated degradation of target RNA. Antisense molecules may also alter protein synthesis by interfering with RNA processing or transport from the nucleus

into the cytoplasm (Mukhopadhyay & Roth, 1996, *Crit. Rev. in Oncogenesis* 7, 151-190).

The antisense molecules described in the art are essentially single-stranded linear oligonucleotides which are known to tolerate a number of mismatches and still form stable hybrids with a target sequence raising the concern of safety and toxicity in organisms. While these molecules are functional, for certain applications, including pharmaceutical compositions, greater specificity, lower toxicity and higher stability may be desirable.

The specificity of oligonucleotides described above may be increased by using the HPH nucleic acid molecule of the present invention which form internal hairpin structure with hydrogen bond interactions. Partial complementarity of these HPH oligonucleotides with target sequences do not allow for efficient opening of the internal hairpin structure of the HPH oligonucleotide resulting in a competition between a hairpin structure and binding to target a sequence. Therefore, hybridization interaction between HPH and target sequences with one or more mismatched sequences, occurs inefficiently, thereby making them inefficient inhibitors of gene expression (see fig. 9).Tyagi & Kramer, 1996, *Nat Biotechnol* 14,303-308; Tyagi & Kramer, 1998, *Nat Biotechnol* 16, 359-363 have shown that oligonucleotides such as molecular beacons which have a 10 base pair or more internal hairpin stems are capable of binding to a target sequence in a highly sequence specific manner in solution. The specific interaction of a hairpin DNA with target RNA was also demonstrated in cells (Kostrikis. *et al.*, 1998 , *Science* 279, 1228-1229) where the hairpin DNA was used to detect the presence of bFGF RNA, these oligonucleotides however were not used to inhibit gene expression.

In addition to the increased specificity, the intramolecular bonding of the hairpin hybridizer molecules may result in increased stability. Hairpin sequences located at the respective ends of the oligonucleotide may increase the stability of these reagents because the lack of unpaired free nucleotides reduces the potential for degradation by exonucleases. End stabilization is currently conferred by chemical modifications

(phosphorothioate linkage etc.) which may itself decrease specificity, and possibly increase cytotoxicity. The increased stability of hairpin-end vector-based ribozyme constructs has already been demonstrated (Thompson *et al.*, 1995, *Nucleic Acids Research* 23, 2259-2268).

The effectiveness of these HPH molecules may be enhanced by the addition of nucleotides which act as substrates for RNase H within the molecule. However, binding of DNA to RNA is not as thermodynamically favorable as an RNA to RNA interaction (Altmann *et al.*, 1996, *Chimia* 50, 168-176). Therefore a molecule with both RNA and DNA nucleotides may be able to bind efficiently as well as promote degradation of the RNA molecule by RNase H. Inoe & Ohtsuka, 1987, *Nucleic Acids Research* 115, 6131, first proposed an oligonucleotide with a central region consisting of oligodeoxynucleotides flanked by 2'-*O*-methyl modified nucleotide regions. The region of oligodeoxynucleotides in such a chimeric molecule is recognized by RNase H when bound to target RNA; and facilitates cleavage of target RNA by RNase H. (Inoe & Ohtsuka, 1987, *FEBS* Lett. 215, 327; Shibahara & Morisava, 1987, *Nucleic Acids Res.* 15, 4403). These chimeric oligonucleotides were proposed to interact with target RNA more stably than an all DNA oligonucleotide. Alternatively, the nucleic acid molecule may function by binding to the target molecule which results in steric hindrance for ribosomal translation. A number of chemical modifications may be utilized with this strategy including insertion of 2'-*O*-methyl modification at every nucleotide in the molecule.

One of the most studied and utilized chemical alteration in oligonucleotides has been backbone modifications such as phosphorothioates, phosphorodithioates, and 5'thiophosphates. Phosphorothioate oligonucleotides are nucleic acid molecules whose phosphodiester linkage has been modified by substituting a sulfur atom in place of an oxygen atom. In addition to increased nuclease resistance, phosphorothioate, phosphorodithioate, and 5'thiophosphates oligonucleotides are substrates for ribonuclease H (RNase H) (Monia, *supra*; Crooke *et al.*, 1995, *Biochem. J.* 3112, 599-608). RNase H is an endonuclease which catalyzes the degradation of RNA in an RNA-

DNA heteroduplex (Hostomsky *et al.*, 1993 in *Nucleases,* Linn *et al.*, eds., Cold Spring Harbor Laboratory Press, NY, 341-376). RNA/DNA heteroduplexes, called Okazaki fragments, are formed naturally during DNA replication. Therefore, the normal function of RNase H is to degrade the RNA portion of the heteroduplex to complete DNA replication. In experiments with *E. Coli* RNase H, the phosphorothioate oligonucleotide activated the enzyme more efficiently (2-5 fold) compared to a standard phosphodiester containing oligonucleotide (Crooke, 1995, *supra*).

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs (*i.e.* HPH molecules) are used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of RNA structure. The molecules of the instant invention were chemically synthesized. Oligodeoxyribonucleotides were synthesized using standard protocols as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19, and is incorporated by reference.

The method of synthesis used for normal RNA including certain enzymatic nucleic acid molecules follows the procedure as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5 µmol scale protocol with a 5 min. coupling step for alkylsilyl protected nucleotides and 2.5 min. coupling step for 2'-*O*-methylated nucleotides. Table I outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 µL of

0.1 M = 16.3 μmol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238 μL of 0.25 M = 59.5 μmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer; detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

Deprotection of the RNA was performed as follows. The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min. After cooling to -20 °C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H_2O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250 μL of a solution of 1.5mL *N*-methylpyrrolidinone, 750 μL TEA and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was prewashed

with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white powder.

The average stepwise coupling yields were >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684).

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247)

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; and *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995 which are both incorporated herein by reference. Sullivan *et al.*, PCT WO 94/02595, further describes the general methods for delivery of enzymatic RNA molecules. These protocols may be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, nucleic acid molecules may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra and Draper *et al.*, PCT WO93/23569 which have been incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

The negatively charged polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the like.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or patient, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation to reach a target cell (*i.e.*, a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body.

Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as the cancer cells.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer an method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, **95**, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, **43**, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, **267**, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, **1238**, 86-90). The long-circulatingliposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, **42**, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392; all of these are incorporated by reference herein). Long-

circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

Alternatively, the nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, 1985 *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci.* USA 83, 399; Scanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992 *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992 *J. Virol*, 66, 1432-41; Weerasinghe *et al.*, 1991 *J.*

Virol, **65**, 5531-4; Ojwang *et al.,* 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen *et al.,* 1992 *Nucleic Acids Res.,* 20, 4581-9; Sarver *et al.,* 1990 *Science* 247, 1222-1225; Thompson *et al.,* 1995 *Nucleic Acids Res.* 23, 2259; Good *et al.,* 1997, *Gene* Therapy, 4, 45; all of the references are hereby incorporated in their totality by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.,* PCT WO 93/23569, and Sullivan *et al.,* PCT WO 94/02595; Ohkawa *et al.,* 1992 *Nucleic Acids Symp. Ser.;* 27, 15-6; Taira *et al.,* 1991, *Nucleic Acids Res.,* 19, 5125-30; Ventura *et al.,* 1993 *Nucleic Acids Res.,* 21, 3249-55; Chowrira *et al.,* 1994 *J. Biol. Chem.* 269, 25856; all of the references are hereby incorporated in their totality by reference herein).

In another aspect of the invention, RNA molecules of the present invention are preferably expressed from transcription units (see for example Couture *et al.,* 1996, *TIG.,* 12, 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to the target mRNA. Delivery of nucleic acid molecule expressing vectors could be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.,* 1996, *TIG.,* 12, 510).

In one aspect the invention features, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention is

disclosed. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

In another aspect the invention features, the expression vector comprises: a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

Transcription of the nucleic acid molecule sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. USA*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules, such as ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc. Natl. Acad. Sci. USA*, 90, 6340-4; L'Huillier *et al.*, 1992 *EMBO J.* 11, 4411-8; Lisziewicz *et al.*, 1993 *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4;

Abstract of the Disclosure

The invention features novel hairpin hybridizer nucleic acid molecules which are able to modulate gene expression.

5

Table I: 2.5 μmol RNA Synthesis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 μL	2.5
S-Ethyl Tetrazole	23.8	238 μL	2.5
Acetic Anhydride	100	233 μL	5 sec
N-Methyl Imidazole	186	233 μL	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery.

Table II. Nucleic Acid Molecules Targeting C-raf With Sequence Mismatches

Seq. I.D. No	Sequence	No. of Mismatches
7	gsgsuscaGsCsGsTsGsCsAsAsGscauug gsascscB	0
8	gsgsuscaGsCsGsTsAsCsAsAsGscauug gsascscB	1
9	gsgsuscaGsCsGsGsAsCsAsAsGscauug gsascscB	2
10	gsgsuscaGsCsAsGsAsTsAsAsGscauug gsascscB	4

Legend

lower case - 2'-*O*-methyl ribonucleotides
uppercase - deoxy-ribonucleotides
s - - phosphorothiote linkage

Table III. HPH Molecules Targeting c-raf RNA with Mismatches

Seq. I.D. No.	Sequence 5' complementary --RNA arm DNA core RNA arm - 3' complementary	Length
1	gscsgsagc gugguca GsCsGsTsGsCsAsAsGs cauugau gcuscsgsc	35
2	gscsgsagc ggguacu TsCsGsGsAsCsAsGsGs uacuuag gcuscsgsc	35
3	csgsasgc gugguca GsCsGsTsGsCsAsAsGs cauugau gcsuscsg	33
4	csgsasgc ggguacu TsCsGsGsAsCsAsGsGs uacuuag gcsuscsg	33
5	csasgsc gugguca GsCsGsTsGsCsAsAsGs cauugau gscsusg	31
6	csasgsc ggguacu TsCsGsGsAsCsAsGsGs uacuuag gscsusg	31

Legend:

lower case - 2'O-methyl ribonucleotides
uppercase - deoxy-ribonucleotides
s - - phosphorothiote linkage
underlined - mismatches.

Figure 1. Hairpin Hybridizer Molecules

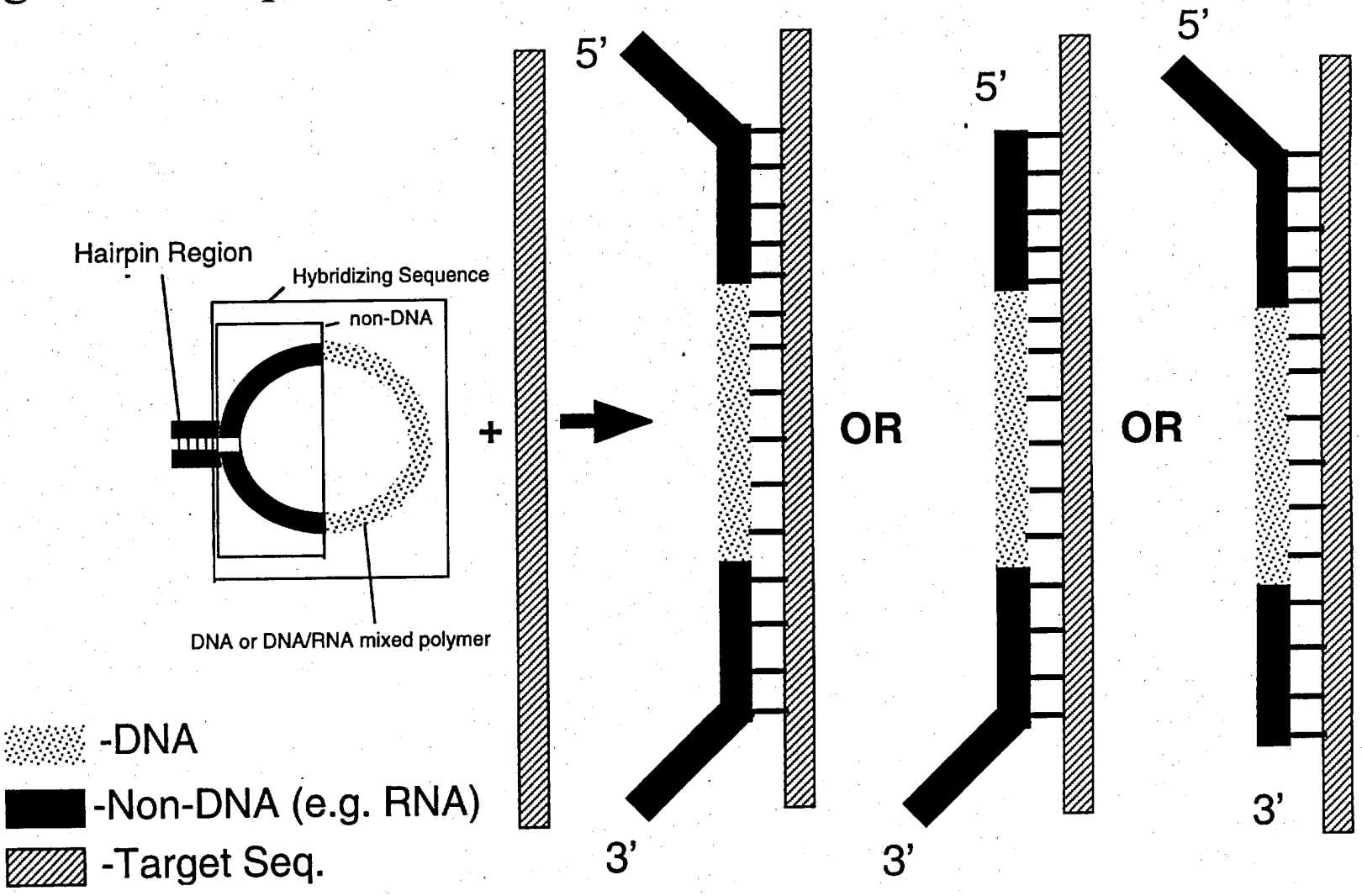
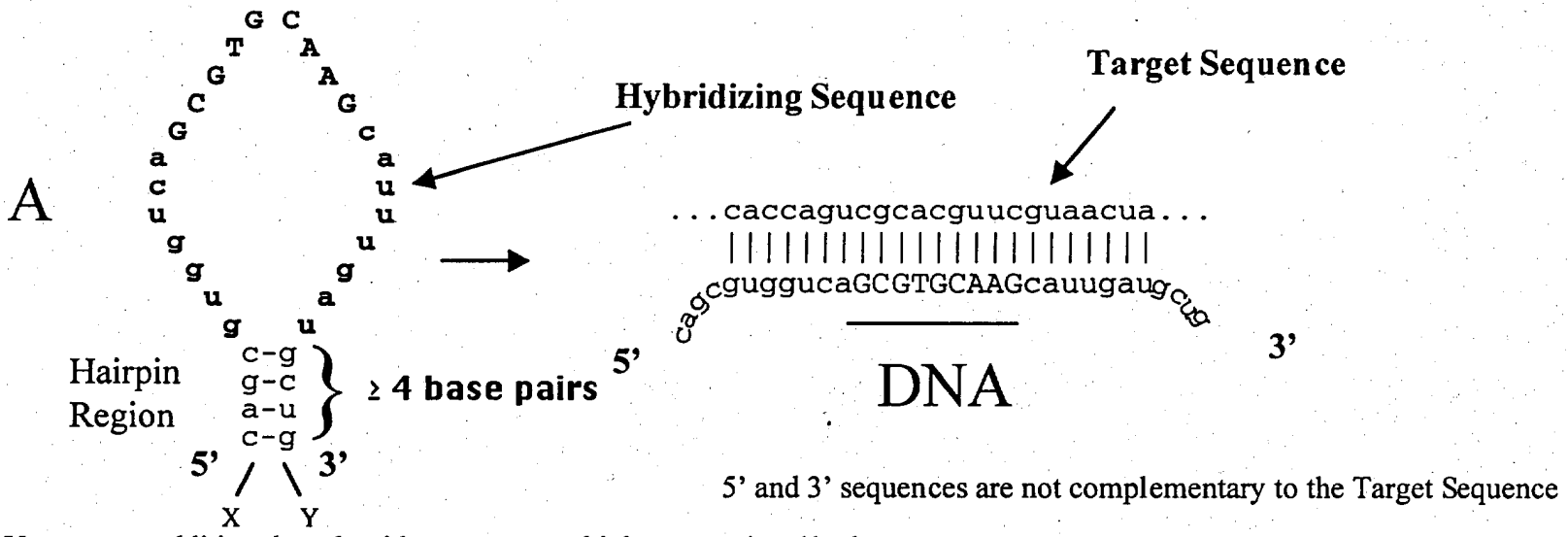


Figure. 2 - Hairpin Hybridizer Molecules



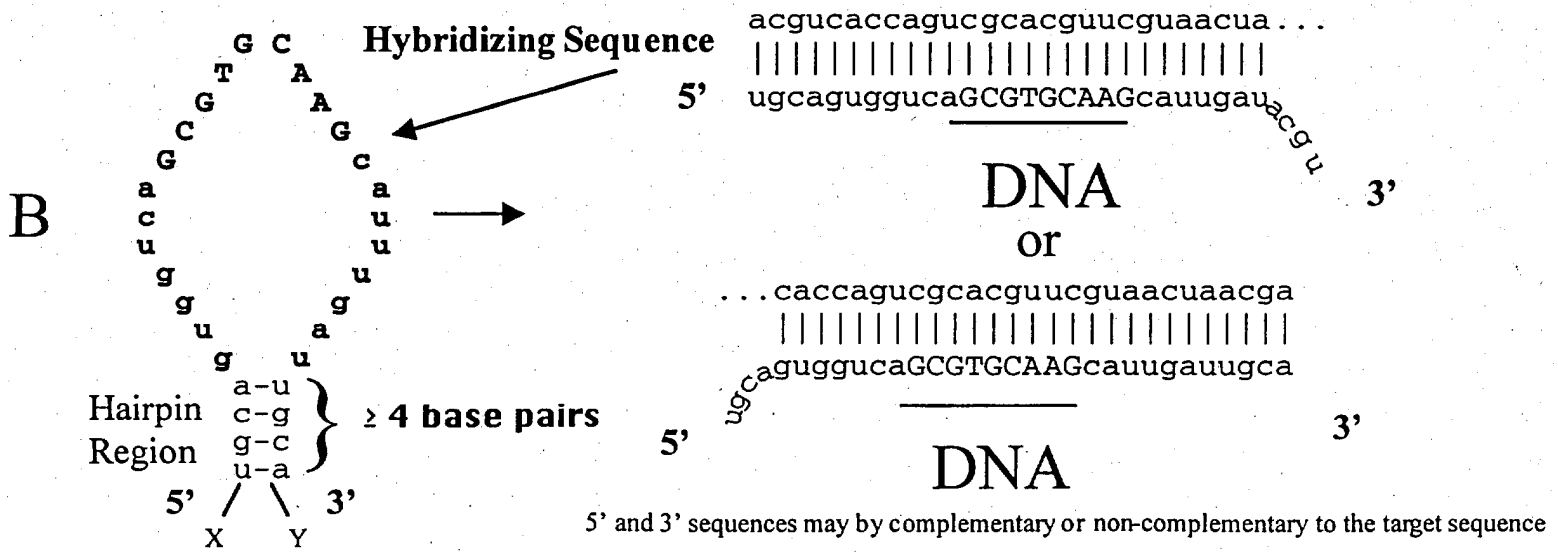


Figure 3. Hairpin Hybridizer Molecule Motifs

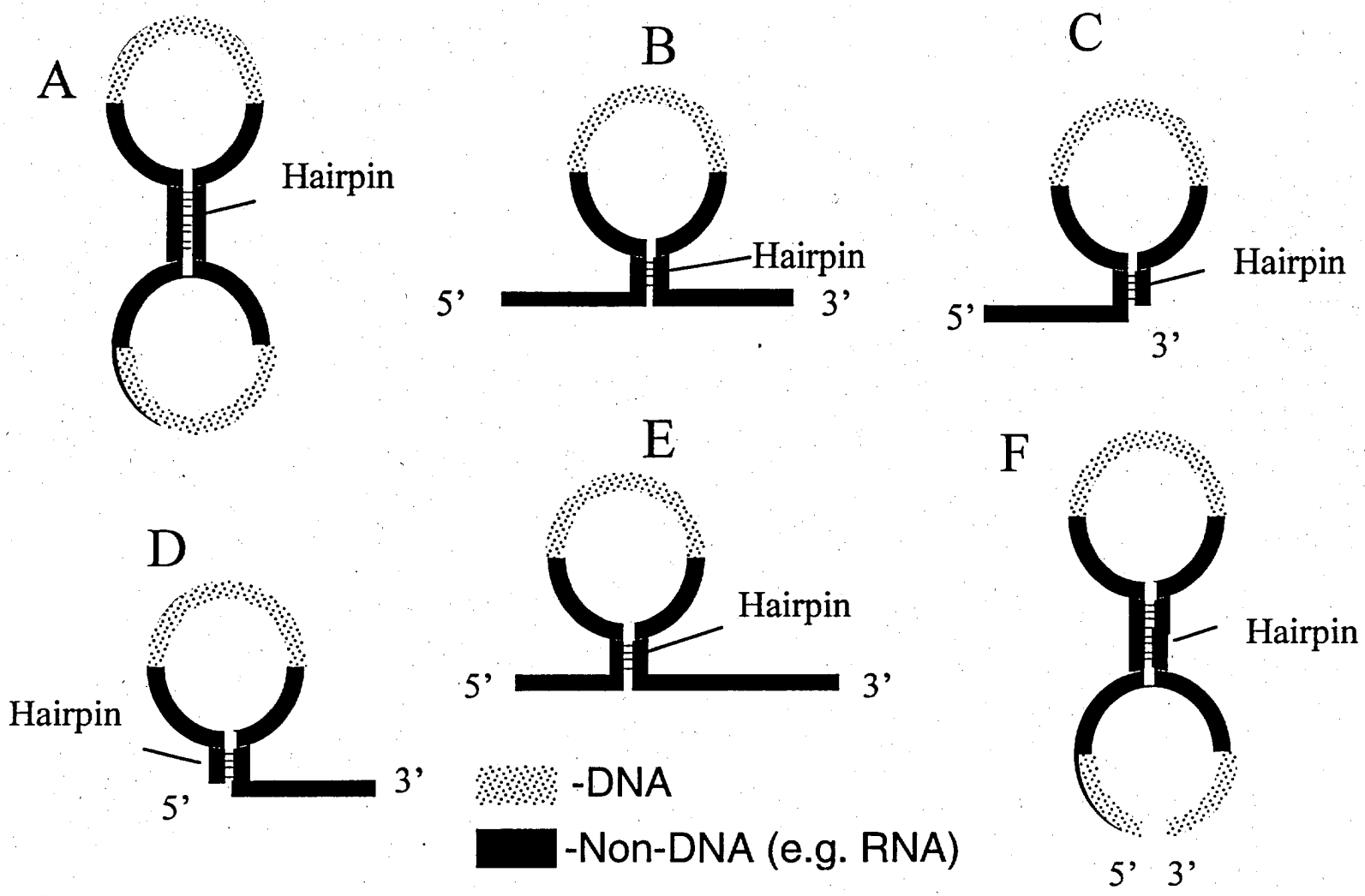


Figure 4. Reduction of c-raf RNA in PC-3 Cells Using a 31mer HPH

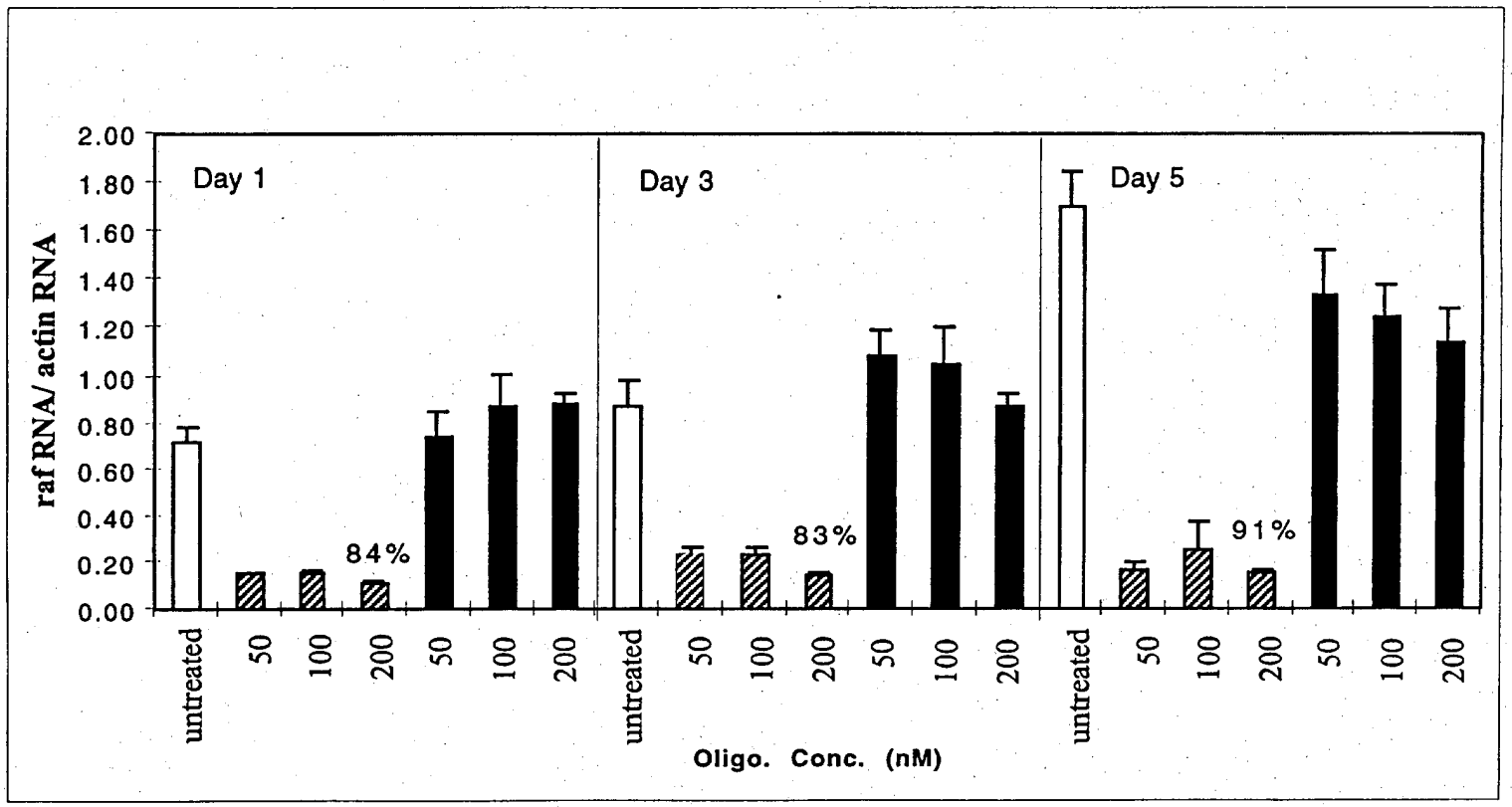


Figure 5. Reduction of c-raf RNA in PC-3 Cells Using a 33mer HPH

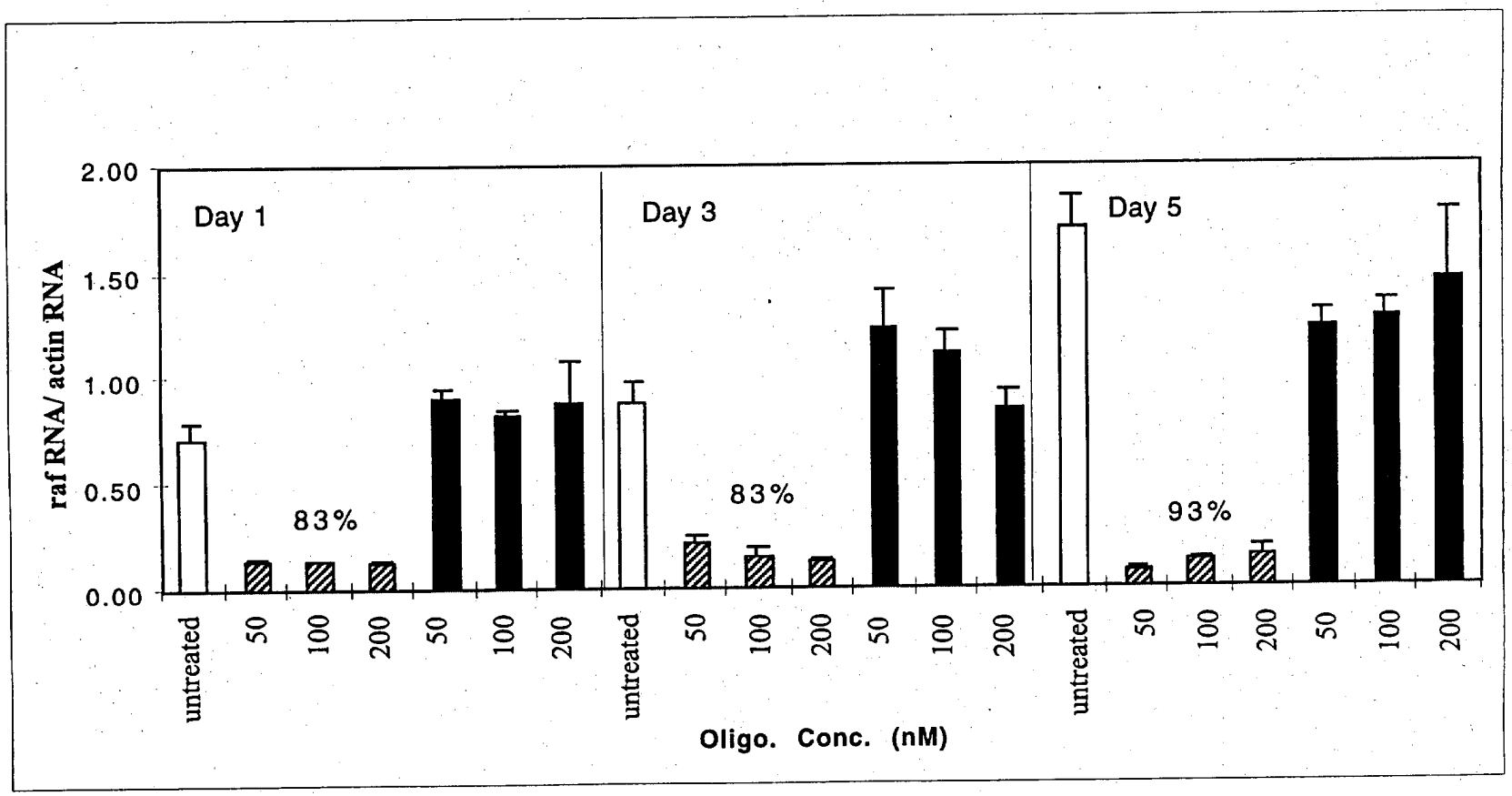


Figure 6. Reduction of c-raf RNA in PC-3 Cells Using a 36mer HPH

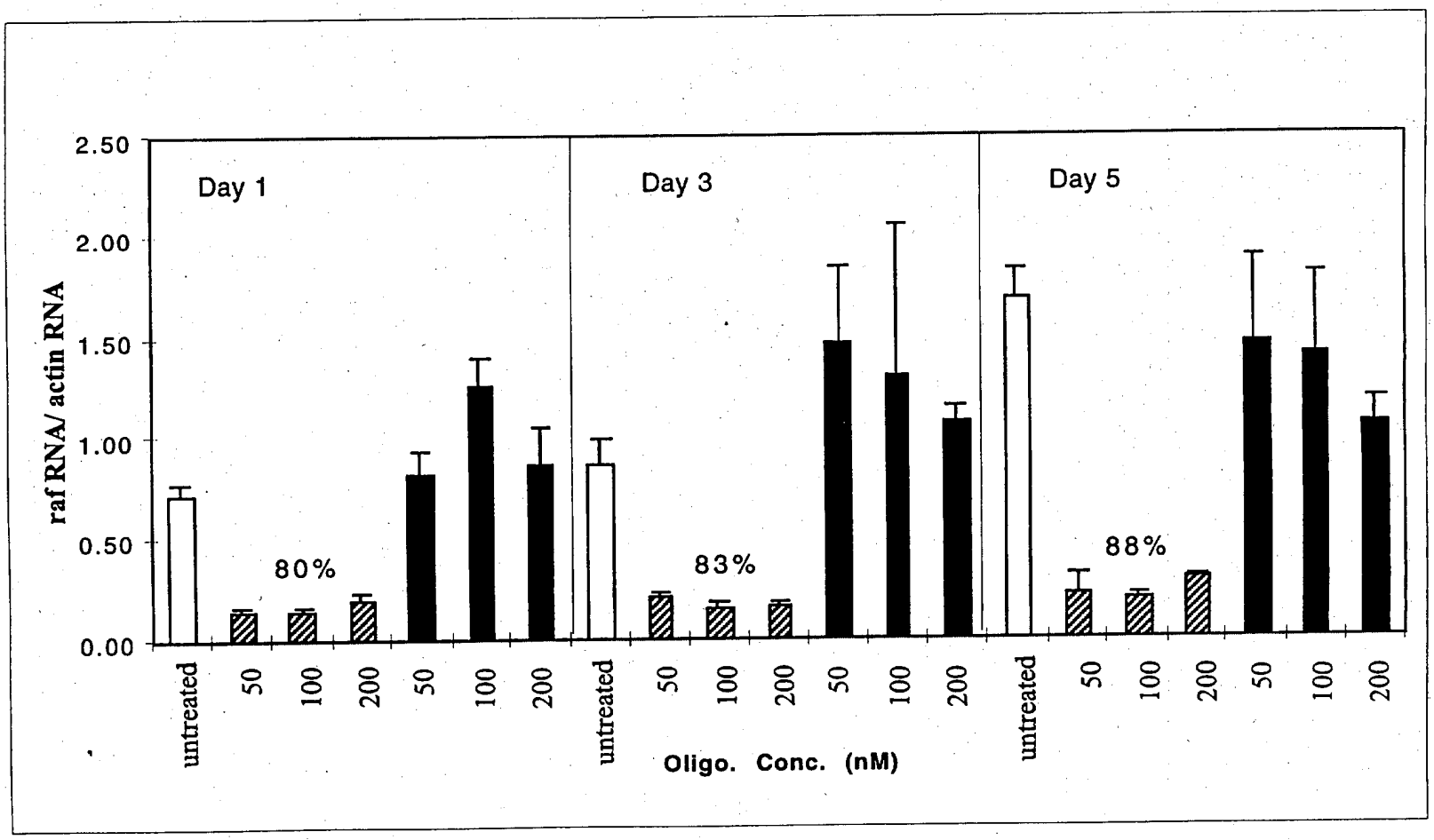


Fig. 7. Reduction of c-raf RNA in PC-3 Cells Using a 31mer HPH

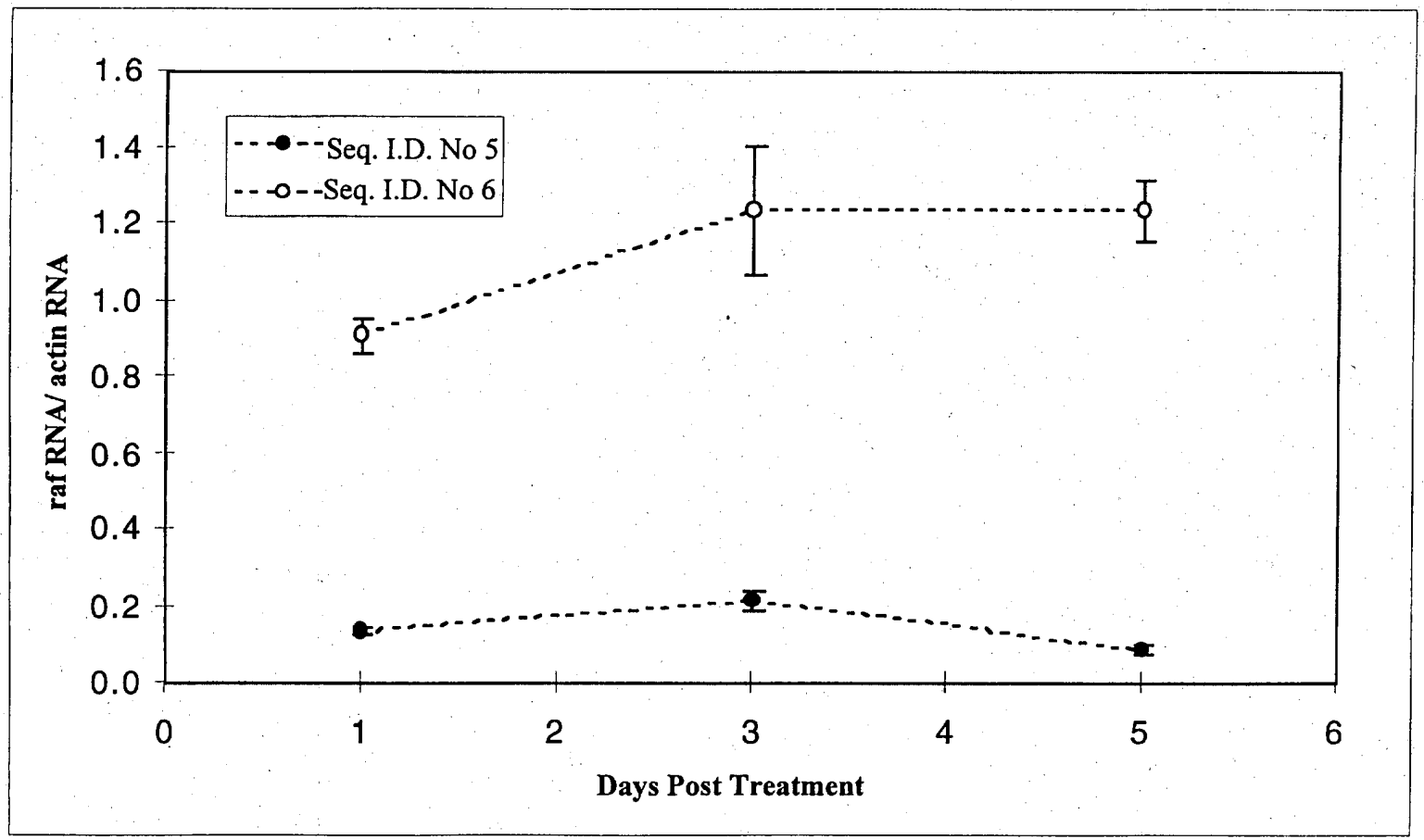


Figure 8. Reduction of c-raf RNA in PC-3 Using a Linear Antisense Molecule

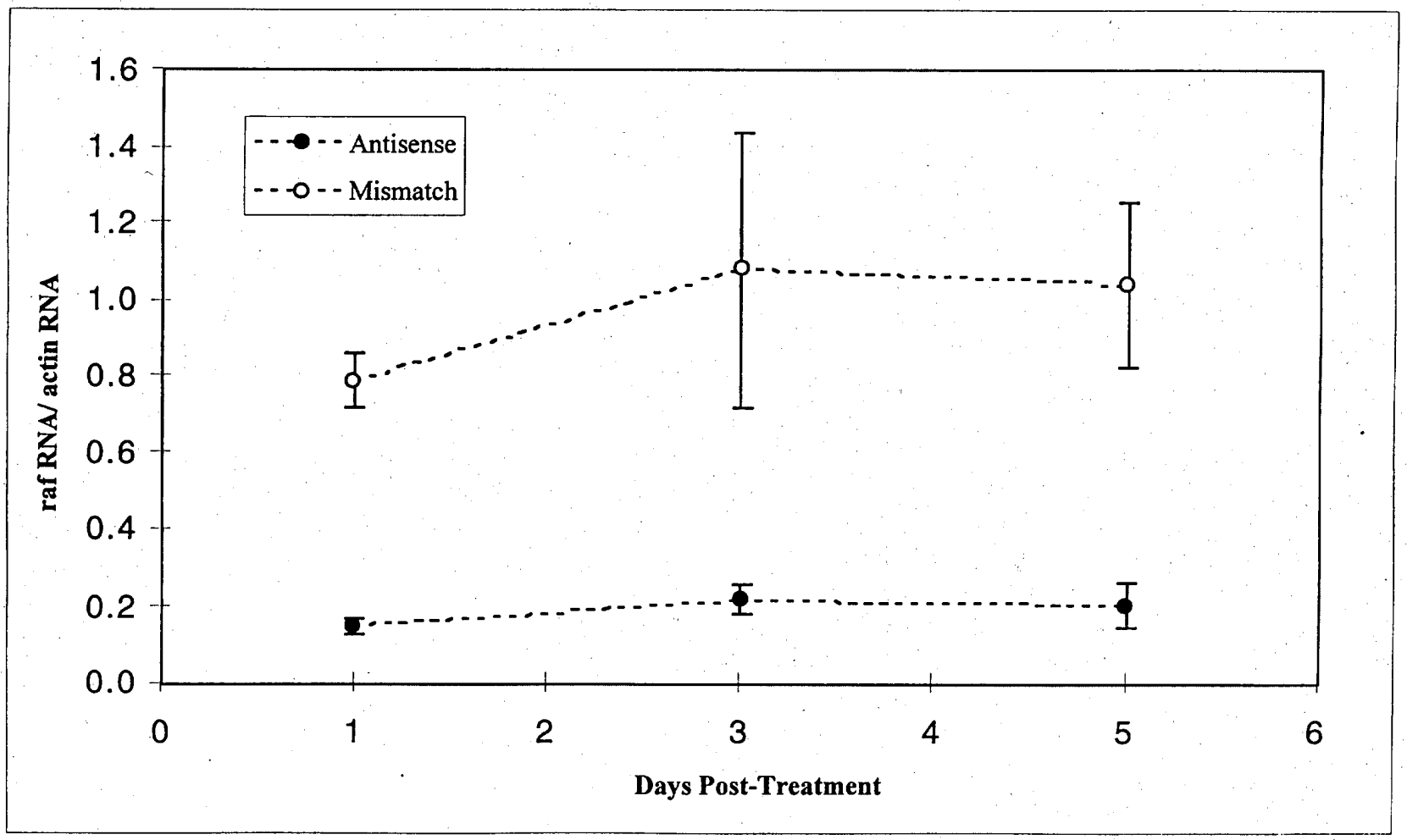


Figure 9. The Effect of Mutations within the Hairpin Nucleic Acid Molecule

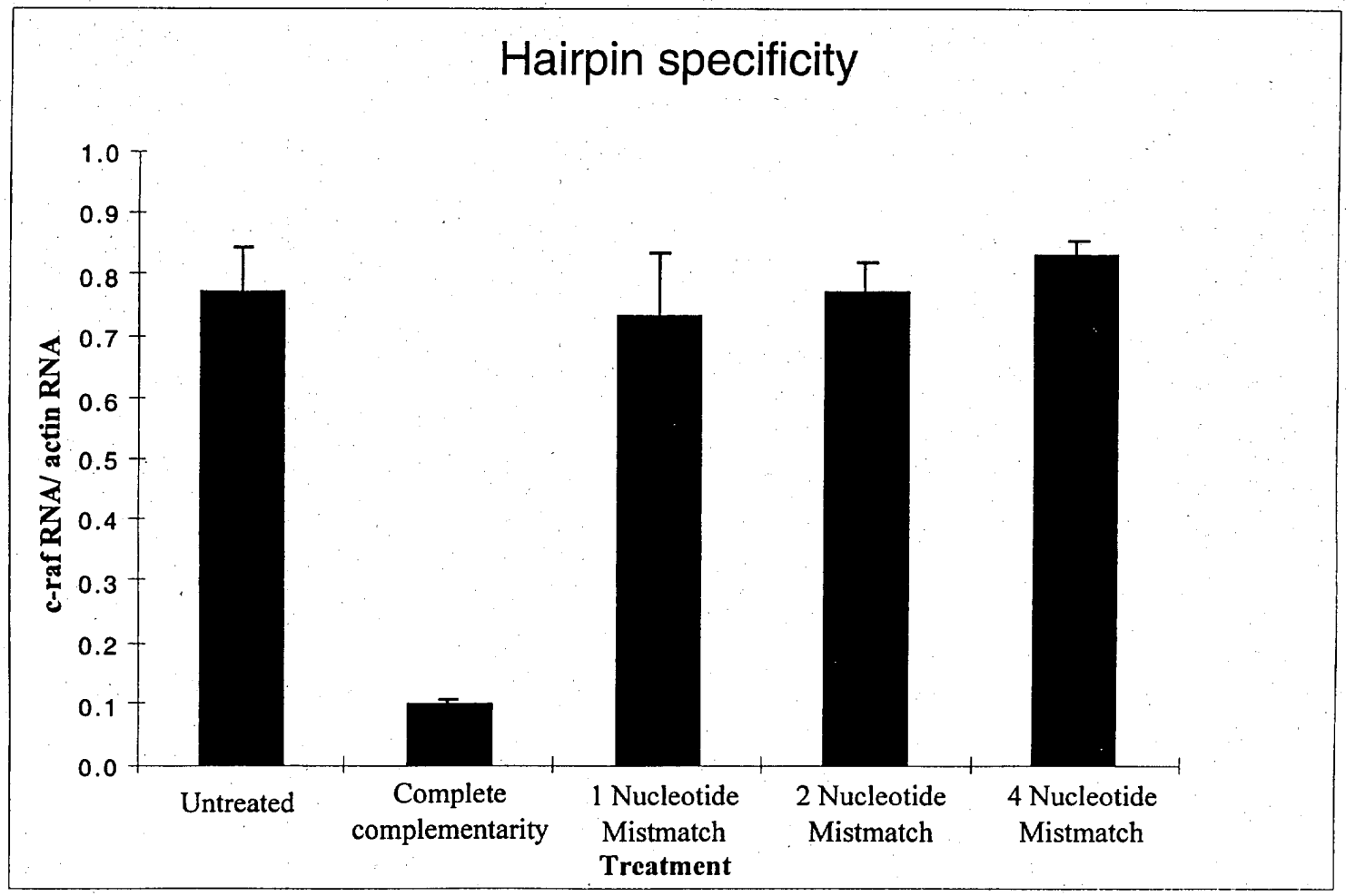
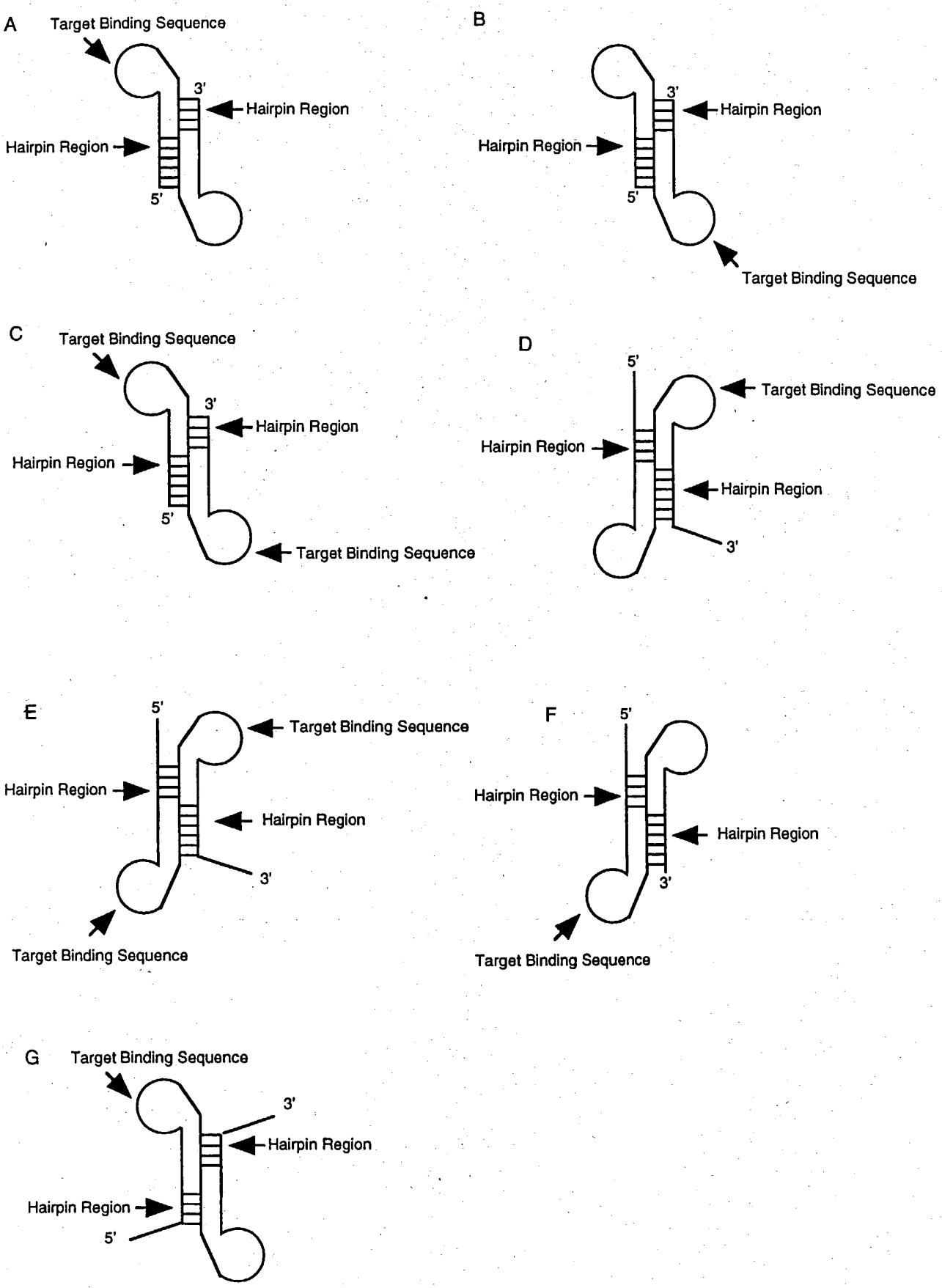


Figure 10: Pseudoknot Hairpin Hybridizer Molecules



Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science,* 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson *et al., supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.* 4, 45; Beigelman *et al.*, International PCT Publication No. *WO 96/18736*; all of these publications are incorporated by reference herein. The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra).*

In yet another aspect the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another preferred embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a gene encoding at least one said nucleic acid molecule; and wherein said

gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

Optimizing Nucleic Acid Molecule Activity

Chemically synthesizing synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases may increase their potency (see *e.g.,* Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al., supra;* all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by reference herein).

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34;

Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996 *Biochemistry* 35, 14090). Sugar modification of enzymatic nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al.* *Nature* 1990, *344*, 565-568; Pieken *et al.* *Science* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, *17*, 334-339; Usman *et al.* *International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the HPH nucleic acid molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, too many of these modifications may cause increased toxicity. Therefore when designing HPH molecules the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity resulting in increased efficacy and higher specificity of these HPH molecules.

Nucleic acid molecules having chemical modifications which maintain or enhance activity are provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. Therapeutic HPH molecules delivered exogenously must optimally be stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, nucleic acid molecules must be resistant to nucleases in order to function as effective intracellular therapeutic agents.

Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19) incorporated by reference herein) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

Target Validation

One of the most challenging tasks in drug discovery is the choice of a therapeutic target. Historically, traditional biochemical and other studies have offered limited information in this regard. However, recent advances in genomics offer the potential to revolutionize both the speed and certainty of therapeutic target identification. Progress in characterizing the genes in the human genome has been very rapid, and it is now estimated that the entire complement of genes in the human genome may be sequenced before the end of this century. However, this mass of information is coming to the scientific world without a road map. Converting pure gene sequence information into a functional understanding of their role in human disease is proving to be a much more difficult problem. Even after a group of genes is associated with a particular disease, the process of validating which genes are appropriate for use as therapeutic targets is often slow and costly. Most companies with genomics activities now have access to myriad partial or full sequences, but do not possess adequate technologies to determine which of those sequences is an appropriate therapeutic target. As a result, only a few genes have been unequivocally identified as the causative agent for a specific disease.

The nucleic acid molecules of the present invention can inhibit gene expression in a highly specific manner by binding to and causing the cleavage of the mRNA corresponding to the gene of interest, and thereby prevent production of the gene product (Christoffersen, *Nature Biotech,* **1997**, 2, 483-484). Appropriate delivery vehicles can be combined with these nucleic acid molecules (including polymers, cationic lipids, liposomes and the like) and delivered to appropriate cell culture or *in vivo* animal disease models as described above. By monitoring inhibition of gene expression and correlation

with phenotypic results, the relative importance of the particular gene sequence to disease pathology can be established. The process may be both fast and highly selective, and allow for the process to be used at any point in the development of the organism. The novel chemical composition of these nucleic acid molecules may allow for added stability and therefore increased efficacy.

Examples

The following are non-limiting examples demonstrating the utility of the nucleic acid molecules of the instant invention. Those in the art will recognize that certain experimental conditions such as temperatures, reaction times, media conditions, transfection reagents, cell types and RNA assays are not meant to be limiting and can be readily modified without significantly altering the protocols.

Example 1: Identification of Potential Binding Sites for the HPH Molecule in the Target Sequence

The sequences of target RNAs were screened for accessible sites using a computer folding algorithm. Regions of the mRNA that did not form secondary folding structures were identified. A more elaborate protocol for identifying appropriate targets may be found in Stinchcomb *et al.*, US Pat. No. 5,646,042 which is incorporated by reference ine its entirety herein.

Example 2: Down-Regulation of c-raf Expression

HPH oligonucleotides targeting exon 11 of the human c-raf gene, with 4-6 complementary nucleotides at the 5' and the 3' end were synthesized using standard protocols (Wincott *et al., supra*). These 5' and 3' sequences were not complementary to the c-raf target. Of the 23 nucleotides complementary to the target sequence, 11 nucleotides in the DNA core and RNA arms were exchanged to generate a control

molecule which lacks the capability to down-regulate c-raf mRNA in a sequence-specific manner. The sequences for nucleic acid molecules used are displayed in table III.

Tissue Culture and Nucleic Acid delivery Protocol: Prostate cancer cells (PC-3) were grown in a growth media consisting of Kaighn's F-12K media, 10% FBS, 1% glutamine, 20 mM HEPES, and 1% pen/strep to sub-confluent densities. A 4X concentration (10 μg/mL) of GSV (Glen Research) was prepared from a 2 mg/mL stock solution as well as a 10μM solution of the nucleic acid molecule of the present invention and its antisense control. Complexes of antisense and GSV were formed in a 96 well plate by channel pipetting in antisense and GSV to form complex solutions which are twice the final concentrations.

Inhibition of c-raf mRNA Using Nucleic acid Molecules of Varying Lengths: Using the cell culture and oligonucleotide delivery method described above, PC-3 cells were treated for 1, 3 or 5 days with lipid-complexed hairpin oligonucleotides. The oligonucleotides used were either 31 (Seq. I.D. No. 12022), 33 (Seq. I.D. No. 12021), or 35 nucleotides (Seq. I.D. No. 12020) in length. Mismatch controls were used to check for non-specific effects and are given above as Seq. I.D. Nos. 12023, 12024, and 12025 for 35, 33, and 31mers, respectively. An untreated control was also tested to determine basal levels of c-raf. PC-3 cells were then harvested with 150 μL of RLT lysis buffer (Qiagen). RNA was purified using Qiagen's instructions and RNA was quantified using Taqman reagents and the 7700 Prism (Perkin Elmer) using the manufacturer's protocol. The ratio of c-raf mRNA over β-actin mRNA was determined by real-time PCR after reverse transcription. Results are shown in Fig. 3-5.

The results show all three molecules demonstrate high levels of reduction of c-raf RNA compared to the mismatch controls regardless of oligonucleotide length. Inhibition levels ranged from 80-93% in PC-3 cells. After each designated time period, PC-3 cells were harvested with 150 μL of RLT lysis buffer (Qiagen). RNA was purified using Qiagen's instructions and RNA was quantified using Taqman reagents and the 7700 Prism

(Perkin Elmer) using the manufacturer's protocol. The ratio of c-raf mRNA over β-actin mRNA was determined by real-time PCR after reverse transcription.

Example 3: Comparison of c-raf inhibition between the Hairpin Hybridizer Molecule and a Linear Antisense Molecule.

To test whether the nucleic acid molecules of the present invention could inhibit c-raf mRNA as well as linear antisense molecules, hairpin and linear antisense molecules were synthesized (Wincott et al., *supra*). Using the cell culture and oligonucleotide delivery method described in example 2, PC-3 cells were treated for 1,3 or 5 days with lipid-complexed hairpin oligonucleotides or a lipid complexed linear antisense molecule. The hairpin molecule (Seq. I.D. No. 5) was 31 nucleotides in length and the results of c-raf inhibition were compared to a mismatch control (Seq. I.D. No. 6). The potency of the antisense molecule was also compared to its mismatch control. After each designated time period, PC-3 cells were harvested with 150 μL of RLT lysis buffer (Qiagen). RNA was purified using Qiagen's instructions and RNA was quantified using Taqman reagents and the 7700 Prism (Perkin Elmer) using the manufacturer's protocol. The ratio of c-raf mRNA over β-actin mRNA was determined by real-time PCR after reverse transcription. The data is given in figures 7 and 8.The HPH molecules significantly reduce the c-raf RNA level while the mismatch molecules did not cause any significant reduction (figure 6, 7).. Similarly, the linear antisense molecule reduced r-raf RNA levels significantly. These experiments demonstrate that the magnitude of c-raf inhibition caused by a hairpin oligonucleotide is comparable with a linear molecule lacking the 5' and 3' hairpin complementary ends

Example 4: Mutation Analysis of the Hairpin Hybridizer Molecule

The nucleic acid molecules of the present invention were designed to bind to c-raf message (table II) with 0, 1, 2, or 4 mismatches within the internal DNA sequence. The

molecules were designed such that the 5'. sequence is complementary to both the 3' sequence as well as the target molecule. These molecules were delivered to PC-3 cells using the cell culture and oligonucleotide delivery protocol described in example 2. The lipid/nucleic acid molecule complexes were added to the cells and allowed to associate for 24 hours. PC-3 cells were then harvested with 150 µL of RLT lysis buffer (Qiagen). RNA was purified using Qiagen's instructions and RNA was quantified using Taqman reagents and the 7700 Prism (Perkin Elmer) using the manufacturer's protocol. The ratio of c-raf mRNA over β-actin mRNA was determined by real-time PCR after reverse transcription. The results are shown in figure 9. Just a single mutation within the HPH nucleic acid molecule is sufficient to destroy the inhibitory effects of the HPH molecule. This shows that the HPH molecules of the present invention are highly sequence specific reagents.

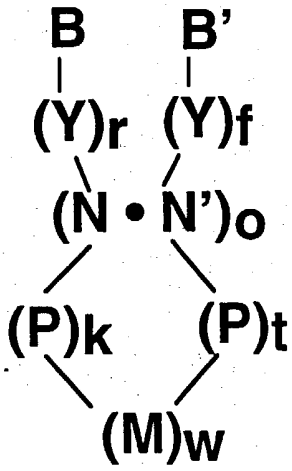
Diagnostic uses

Nucleic acid molecules of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of specific RNAs in a cell. The close relationship between antisense activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple nucleic acid molecules described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Inhibition of target RNAs with nucleic acid molecules may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple nucleic acid molecules targeted to different genes, nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of nucleic acid molecules and/or other chemical or biological molecules). Other *in vitro* uses of nucleic acid molecules of this invention are well known in the art, and include detection of the presence of RNAs related to various conditions.

Other embodiments are within the following claims.

Claims

1. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence expression, wherein said hairpin hybridizer nucleic acid molecule consists of the formula I:



wherein, each P, Y, N, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides; N' is a nucleotide complementary to N; o is an integer greater than or equal to 3; w is an integer greater than or equal to 4; k and t are independently zero or an integer greater than or equal to 3; wherein when t or k is independently 3 or greater, at least one said P is not a 2'-H containing nucleotide; each said $(P)_t$ and $(P)_k$ includes internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and methylphosphonate; $(M)_w$ is an oligonucleotide including one or more internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate, and

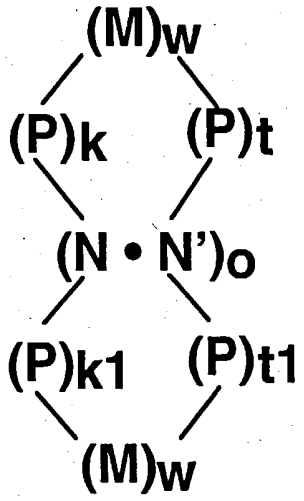
phosphorodithioate linkage; wherein at least one or more of each said (P)t, (P)k, and $(M)_w$ is an oligonucleotide is of sufficient length to stably interact with the target sequence; r and f are independently an integer greater than or equal to zero; each B and B' independently represent, a cap structure which may independently be present or absent; and _____ represents a chemical linkage.

2. The method of Claim 1, wherein, k in said hairpin hybridizer nucleic acid molecule is less than 100.
3. The method of Claim 2, wherein, k in said hairpin hybridizer nucleic acid molecule is selected from the group consisting of 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, and 20.
4. The method of claim 1, wherein, t in said hairpin hybridizer nucleic acid molecule is less than 100.
5. The method of claim 4, wherein, t in said hairpin hybridizer nucleic acid molecule is selected from the group consisting of 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, and 20.
6. The method of claim 1, wherein, k and t in said hairpin hybridizer nucleic acid molecule are of the same length.
7. The method of claim 1, wherein, k and t in said hairpin hybridizer nucleic acid molecule are of different lengths.
8. The method of claim 1, wherein w in said hairpin hybridizer nucleic acid molecule is less than 100.
9. The method of claim 8, wherein w in said hairpin hybridizer nucleic acid molecule is selected from the group consisting of 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, and 20.
10. The method of claim 1, wherein the t, k, and w in said hairpin hybridizer nucleic acid molecule are of same length.
11. The method of nucleic acid molecule of claim 1, wherein t, k, and w in said hairpin hybridizer nucleic acid molecule are of different length.
12. The method of claim 1, wherein the target sequence is selected from the group consisting of RNA, DNA and RNA/DNA mixed polymers.

13. The method of claim 1, wherein r and f in said hairpin hybridizer nucleic acid molecule are independently selected from the group consisting of 1, 2, 3, 4, 5, 10, and 15.

14. The method of claim 1, wherein the chemical linkage in said hairpin hybridizer nucleic acid molecule is selected from the group consisting of phosphate ester linkage, amide linkage, phosphorothioate, 5'-thiopohisphate, methylphosphonate, and phosphorodithioate.

15. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence function, wherein said hairpin hybridizer nucleic acid molecule consists of the formula **II**:



wherein, each P, N, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; o is an integer greater than or equal to 3; w is an integer greater than or equal to 4; k, t, k_1 and t_1 are independently zero or an integer greater than or equal to 3; wherein when t, k, t1,

or k1 is independently 3 or greater at least one said P is not a 2'-H containing nucleotide; each said $(P)_t$, $(P)_k$, $(P)_{t1}$, and $(P)_{k1}$ independently include internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and methylphosphonate; $(M)_w$ is an oligonucleotide sequence including one or more internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate, and phosphorodithioate linkers; wherein at least one or more of each said $(P)_t$, $(P)_k$, $(P)_{t1}$, $(P)_{k1}$and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with the target sequence; and _____ represents a chemical linkage.

16. The method of claim 15, wherein each k, t, k1, t1 and w in said hairpin hybridizer molecule is less than 100.

17. The method of claim 16, wherein each k, t, k1, t1 and w in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, and 20.

18. The method of claim 15, wherein, k and t in said hairpin hybridizer nucleic acid molecule are of same length.

19. The method of claim 15, wherein, k and t in said hairpin hybridizer nucleic acid molecule are of different length.

20. The method of claim 15, wherein, t, k, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

21. The method of claim 15, wherein, t, k, and w in said hairpin hybridizer nucleic acid molecule are of different length.

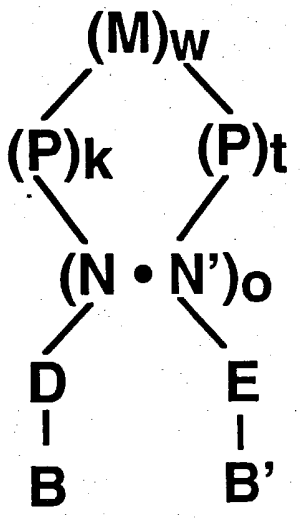
22. The method of claim 15, wherein, t1, k1, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

23. The method of claim 15, wherein, t1, k1, and w in said hairpin hybridizer nucleic acid molecule are of different length.

24. The method of claim 15, wherein the target sequence is selected from the group consisting of an RNA, DNA and RNA/DNA mixed polymer.

25. The method of claim 15, wherein said chemical linkage in said hairpin hybridizer nucleic acid molecule is selected from the group consisting of phosphate ester linkage, amide linkage, phosphorothioate, 5'-thiophosphate, methylphosphonate, and phosphorodithioate.

26. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence function, wherein said hairpin hybridizer nucleic acid molecule consists of formula III:



wherein, each P, N, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; o is an integer greater than or equal to 3; w is an integer greater than or equal to 4; k and t independently are zero or an integer greater than or equal to 3; wherein when t and k are independently 3 or greater at least one said P is not a 2'-H containing nucleotide; each said (P)t and (P)k includes internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and

methylphosphonate; (M)w is an oligonucleotide sequence including on or more internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate and phosphorodithioate; D and E are oligonucleotides independently of length greater than or equal to 4; wherein at least one or more of each said $(P)_t$, $(P)_k$, $(M)_w$, D and E is independently an oligonucleotide of sufficient length to stably interact independently with a target nucleic acid sequence; B and B' independently represent a cap structure which may be present or absent; and _____ represents a chemicallinkage.

27. The method of claim 26, wherein each k, t, and w in said hairpin hybridizer nucleic acid molecule is independently less than 100.

28. The method of claim 27, wherein each k, t, and w in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 4, 5, 6, 7, 8, 9, 10,11, 12, 15, and 20.

29. The method of claim 26, wherein k and t in said hairpin hybridizer nucleic acid molecule are of the same length.

30. The method of claim 26, wherein k and t in said hairpin hybridizer nucleic acid molecule are of different length.

31. The method of claim 26, wherein each t, k, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

32. The method of claim 26, wherein each t, k, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

33. The method of claim 26, wherein the target nucleic acid sequence is selected from the group consisting of RNA, DNA and RNA/DNA mixed polymer.

34. The method of claim 26, wherein each D and E oligonucleotides is independently less than 100 nucleotides in length.

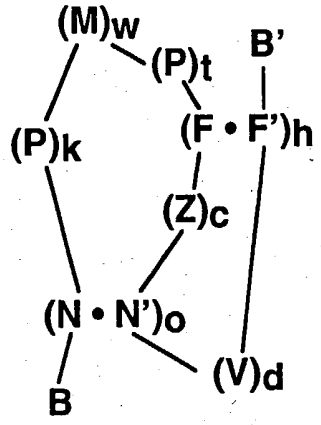
35. The method of claim 26, wherein the length of each D and E oligonucleotides is independently selected from the group consisting of 6, 7, 8, 9, 10, 11, 12, 15, 20, and 30 nucleotides.

36. The method of claim 26, wherein D and E oligonucleotides in said hairpin hybridizer nucleic acid molecule in said hairpin hybridizer nucleic acid molecule are of the same length.

37. The method of claim 26, wherein said oligonucleotides, said D and said E oligonucleotides in said hairpin hybridizer nucleic acid molecule are of different length.

38. The method of claim 26, wherein said chemical linkage is selected from the group consisting of phosphate ester linkage, amide linkage, phosphorothioate, and phosphorodithioate.

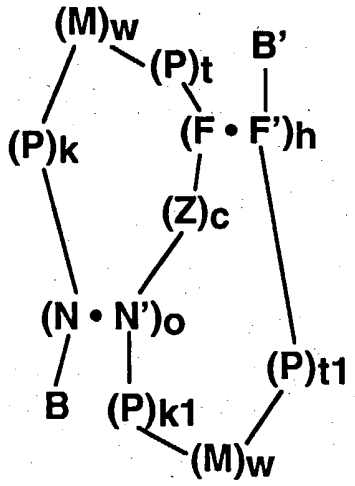
39. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence function, wherein said hairpin hybridizer nucleic acid molecule consists of formula V:



wherein, each P, N, F, V, Z, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide

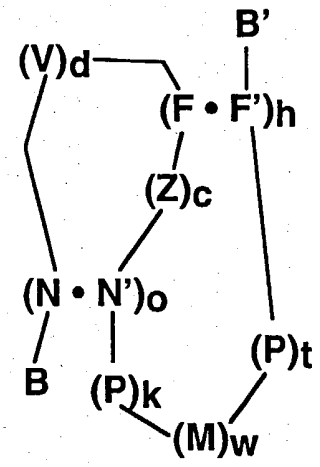
complementary to F; o is an integer greater than or equal to 3; w is an integer greater than or equal to 4; d is an integer greater than or equal to 3; h is an integer greater than or equal to 2; c is an integer greater than or equal to 0; k and t is independently, zero or an integer greater than or equal to 3; wherein when t and k are independently 3 or greater, at least one said P is not a 2'-H containing nucleotide; each $(P)_t$,$(P)_k$, $(V)_d$ and $(Z)_C$ includes internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and methylphosphonate; (M)w is an oligonucleotide including one or more internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate and phosphorodithioate linkage; wherein at least one or more of each said (P)t, (P)k, and (M)w is an oligonucleotide of sufficient length to stably interact independently with a target sequence; each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage.

40. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence function, wherein said hairpin hybridizer nucleic acid molecule consists of formula VI:



wherein, each P, N, F, Z and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide complementary to F; o is an integer greater than or equal to 3; k and t is independently, zero or an integer greater than or equal to 3; k1 and t1 are independently zero or an integer greater than or equal to 3; w is an integer greater than or equal to 4; h is an integer greater than or equal to 2; c is an integer greater than or equal to 0; wherein when t, k, t1, or k1 is independently 3 or greater at least one said P is not a 2'-H containing nucleotide; each $(P)_t$,$(P)_k$, $(P)_{t1}$,$(P)_{k1}$, and $(Z)_C$ includes internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and methylphosphonate; $(M)_w$ is an oligonucleotide including one or more inter-nucleotide linkerages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate and phosphorodithioate linkage; wherein at least one or more of each said $(P)_t$, $(P)_k$, $(P)_{t1}$,$(P)_{k1}$, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target nucleic acid molecule; each B and B' independently represents a cap structure which may independently be present or absent; and _____ represents a chemicallinkage.

41. A method of modulating the function of a target sequence in a cell comprising the step of contacting said cell with a hairpin hybridizer (HPH) nucleic acid molecule under conditions suitable for the modulation of said target sequence function, wherein said hairpin hybridizer nucleic acid molecule consists of formula VII:



wherein, each P, N, F, V, Z, and M represents independently a nucleotide which may be same or different; • indicates hydrogen bond formation between two adjacent nucleotides, N' is a nucleotide complementary to N; F' is a nucleotide complementary to F; o is an integer greater than or equal to 3; w is an integer greater than or equal to 4; d is an integer greater than or equal to 3; h is an integer greater than or equal to 2; c is an integer greater than or equal to 0; k and t is independently, zero or an integer greater than or equal to 3; wherein when t and k are independently 3 or greater, at least one said P is not a 2'-H containing nucleotide; each $(P)_t$,$(P)_k$, $(V)_d$ and $(Z)_C$ includes internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, 5'thiophosphate, and methylphosphonate; (M)w is an oligonucleotide including one or more internucleotide linkages selected from the group consisting of phosphodiester, phosphorothioate, 5'thiophosphate, methylphosphonate and phosphorodithioate linkage; wherein at least one or more of each said $(P)_t$, $(P)_k$, and $(M)_w$ is an oligonucleotide of sufficient length to stably interact independently with a target sequence; each B and B' independently

represents a cap structure which may independently be present or absent; and _____ represents a chemical linkage.

42. The method of any of claims 39-41, wherein each k, t, and w in said hairpin hybridizer nucleic acid molecule is independently less than 100.

43. The method of claims 42, wherein each k, t, and w in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 4, 5, 6, 7, 8, 9, 10,11, 12, 15, and 20.

44. The method of any of claims 39 or 41 wherein d in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, and 18.

45. The method of any of claims 39-41, wherein h in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, and 9.

46. The method of any of claims 39-41, wherein c in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, and 18.

47. The method of any of claims 39-41, wherein o in said hairpin hybridizer nucleic acid molecule is selected from the group consisting to 4, 5, 6, 7, 8 and 9.

48. The method of any of claims 39-41, wherein k and t in said hairpin hybridizer nucleic acid molecule are of the same length.

49. The method of any of claims 39-41, wherein k and t in said hairpin hybridizer nucleic acid molecule are of different length.

50. The method of any of claims 39-41, wherein each t, k, and w in said hairpin hybridizer nucleic acid molecule are of different length.

51. The method of any of claims 39-41, wherein each t, k, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

52. The method of any of claims 39-41, wherein the target sequence is selected from the group consisting of RNA, DNA and RNA/DNA mixed polymer.

53. The method of any of claims 39-41, wherein said chemical linkage is selected from the group consisting of phosphate ester linkage, amide linkage, phosphorothioate, 5'-thiophosphate, methylphosphonate, and phosphorodithioate.

54. The method of claim 40, wherein each k1 and t1 in said hairpin hybridizer nucleic acid molecule is independently less than 100.

55. The method of claim 40,wherein each k1and t1 in said hairpin hybridizer nucleic acid molecule is independently selected from the group consisting of 4, 5, 6, 7, 8, 9, 10,11, 12, 15, and 20.

56. The method of claim 40,wherein each t1, k1, and w in said hairpin hybridizer nucleic acid molecule are of different length.

57. The method of claims 40,wherein each t1, k1, and w in said hairpin hybridizer nucleic acid molecule are of the same length.

58. The method of any of claims 39-41, wherein said F portion of the $(F•F')_h$ in the HPH nucleic acid molecule is complementary to a portion of said target sequence.

59. The method of any of claims 39-41, wherein said F' portion of the $(F•F')_h$ in the HPH nucleic acid molecule is complementary to said target sequence.

60. The method of any of claims 39-41, wherein said F and said F' portion of the $(F•F')_h$ in the HPH nucleic acid molecule is independently complementary to said target sequence.

61. The method of any of claims 1, 15, 26, or 39-41, wherein said N portion of the (N•N')o in the HPH nucleic acid molecule is complementary to a portion of said target sequence.

62. The method of any of claims 1, 15, 26, or 39-41, wherein said N' portion of the (N•N')o in the HPH nucleic acid molecule is complementary to said target sequence.

63. The method of any of claims 1, 15, 26, or 39-41, wherein said N and said N' portion of the (N•N')o in the HPH nucleic acid molecule is independently complementary to said target sequence.

64. The method of any of claims 39-41 wherein, said $(Z)_c$ in the HPH nucleic acid molecule is complementary to a target sequence.

65. The method of any of claims 1, 15, 26, or 39-41, wherein each said $(P)_k$, $(P)_t$, $(N•N')_o$, and $(M)_w$, in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

66. The method of claim 1, wherein each said $(Y)_r$ and $(Y)_f$ in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

67. The method of claim 15 or 40 wherein each said $(P)_{k1}$, and $(P)_{t1}$, in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

68. The method of claim 26, wherein each said D and E in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

69. The method of any of claims 39-41, wherein each said $(Z)_c$, and $(F•F')_h$, in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

70. The method of any of claims 39 or 41 wherein each said $(V)_d$, in the HPH nucleic acid molecule comprises independently a nucleotide modification selected from the group consisting of: 2'-O-methyl, 2'-O-allyl, 2'-O-methylthiomethyl, L-nucleotides; 2'-C-allyl; 1-5-Anhydrohexytol; 2,6-diaminopurine; 2'-fluoro; 2'-deoxy-2'-amino; 2'-(N-alanyl) amino; 2'-(N-phenylalanyl)amino; 2'-deoxy-2'-(N-_-alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino; 2'-Deoxy-2'-(N-histidyl) amino; 6-methyl uridine; 5-methyl cytidine; 2'-(N-_-carboxamidine-_-alanyl) amino-2'-deoxy-nucleotide; and xylofuranosyl.

71. The method of any of claims 1, 26, or 39-41, wherein said B' when present, selected from the group consisting of: inverted abasic residue; 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides;

modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; and methylphosphonate moiety.

72. The method of any of claims 1, 26, or 39-41, wherein said nucleic acid molecule comprises a 3'-3' linked inverted abasic moiety at said 3' end.

73. The method of any of claims 1, 26, or 39-41, wherein said B when present, is selected from the group consisting of: 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moeity; 5'-5'-inverted abasic moeity; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties.

74. The method of any of claims 1, 15, 26, or 39-41 wherein said cell is a mammalian cell.

75. The method of any of claims 1, 15, 26, or 39-41 wherein said cell is a plant cell.

76. The method of any of claims 1, 15, 26, or 39-41 wherein said cell is a bacterial cell

77. The method of any of claims 1, 15, 26, or 39-41 wherein said cell is a microbial cell.

78. The method of any of claims 1, 15, 26, or 39-41 wherein said cell is a fungal cell.

79. The mammalian cell of claim 74, wherein said mammalian cell is a human cell.

80. The method of any of claims 1, 15, 26, or 39-41, wherein said HPH nucleic acid molecule is chemically synthesized.

81. The method of any of claims 1, 15, 26, or 39-41, wherein said HPH is a pharmaceutical composition .

82. The method of any of claims 1, 15, 26, or 39-41, wherein said modulation of function is the modulation of the phenotype of the cell.